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THE OXYGEN CONSUMPTION OF NORMAL RAT LIVER SLICES IN SERUM AND IN LYMPH TAKEN FROM THE LEGS BEFORE AND AFTER SEVERE BURNS

BY JYTTE MUUS AND ESTHER HARDENBERGH

*(From the Department of Physiology, Harvard School of Public Health, Boston, and the
Department of Physiology, Mount Holyoke College, South Hadley)*

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The following experiments were carried out as part of an investigation on burns which is in progress at the Harvard School of Public Health (1-3).

The toxic theory of burns, although in disfavor at present, has never been conclusively disproved and there is still much evidence to support it. It was decided to attempt to find some criterion for the toxicity of the exudate from burned regions. Friend and Hastings (4) had suggested that the oxygen consumption of tissue slices suspended in serum might be sensitive to toxic substances present in the serum. A series of experiments was therefore done comparing the oxygen consumption of rat liver slices suspended in lymph or serum from normal calves with that of slices from the same liver in lymph or serum collected some time after burning.

We are not aware of any similar studies on burns. Beecher and Craig (5) studied the oxygen consumption of tissues from animals in shock due to hemorrhage and found it not significantly different from normal. Their purpose was to demonstrate possible changes in the metabolizing tissue, while ours is to demonstrate changes in metabolism due to changes in the medium. If such changes were the result of the release of substances from the injured tissue, it would seem that the lymph collected from the burned area would be the best place to demonstrate their presence.

In our opinion, the results do not indicate the presence of a "toxic" substance in the above sense. We were inclined to expect the effect, if any, to be a decrease in metabolism. Actually a higher oxygen consumption was observed when lymph collected after burning served as a medium than when normal lymph was used. An attempt to obtain some information about the nature of the substance or substances responsible for this increase in metabolism was possible only in the last three experiments. In the earlier experiments most of the lymph was used for analyses of some of the nitrogenous constituents (2) and for electrophoretic studies (3).

EXPERIMENTAL

Calves weighing between 125 and 200 pounds were used. A single experiment on a dog is also included. All animals were kept under complete

anesthesia with nembutal, and those which did not die from the burns were sacrificed at the end of the experiment. Details of the general procedure for lymph collection from calves, length and method of burning, etc., have been published elsewhere (2). To facilitate comparison with other observations on the same animals, the calves are numbered as in previous publications (2, 3). In all cases lymph was collected from both front legs without anticoagulant for some hours; then one leg was burned by immersion in boiling water for 3 to 3½ minutes.¹ Lymph collection was continued over a period of from 10 to 22 hours. Blood was drawn from the jugular vein just before the burn and at the end of the experiment. Calf 10 died before a final blood sample was secured.

The oxygen consumption was measured by the direct Warburg technique. The serum and lymph were freed from carbon dioxide by the method of Friend and Hastings (4). The pH was adjusted to between 7.2 and 7.4, the two samples to be compared not differing more than 0.1 pH, and the pH was again checked at the end of the experiment. 2 cc. samples and 0.1 cc. of 4 per cent glucose were placed in each of the vessels of the Erlenmeyer type. Slices of liver from healthy, well fed rats were then introduced into the vessels after first being washed in Ringer-phosphate solution (6). The determinations were made in duplicate. A vessel containing the serum or lymph without added tissue was set up simultaneously to exclude the possibility that bacterial contamination was responsible for part of the oxygen consumption. The temperature of the bath was 37°.

The amount of lymph that could be secured during an experiment was so small that, even in the experiments in which all the lymph was available and the effect produced was large enough to warrant fractionations, methods had to be such that they could be employed on a small scale. This was especially true of the normal lymph, which had to be carried through the same procedures as the control. The preliminary period of collecting was 3 to 4 hours and it was inadvisable to extend it much, particularly because of the danger of coagulation in the lymph vessel when no anticoagulant was used.

Ultrafiltrates were made from the lymph after the carbon dioxide had been removed and the pH adjusted to 7.4. Visking tubing and an ultrafiltration tube similar to that described by Coolidge (7) were used. The ultrafiltrates were always more alkaline, and acid was added to adjust the pH to 7.4.

In the last experiment 5 cc. of ultrafiltrate, made acid with 1 cc. of

¹ In most cases, for reasons not connected with this paper, one more burn (sometimes two) was inflicted elsewhere from 1 to 4 hours later. This apparently had no effect on the results with lymph from the leg first burned, but might influence the results with serum.

1 M phosphoric acid, were subjected to continuous extraction for 3 hours with 40 cc. of peroxide-free ether, the ether being refluxed and made to flow through the solution. The ether was evaporated in a stream of nitrogen, and the extract dissolved in 5 cc. of Ringer-phosphate solution and neutralized with sodium hydroxide to pH 7.4. The extracted solution was neutralized with solid calcium carbonate, the carbon dioxide removed with nitrogen, and the calcium phosphate centrifuged off.

Mercuric acetate precipitation was carried out on samples of the same ultrafiltrates by adding 1 cc. of 20 per cent mercuric acetate to 5 cc. of ultrafiltrate. The precipitate was separated by centrifugation, washed with water, and suspended in 4.5 cc. of Ringer-phosphate solution. The mercury was removed with hydrogen sulfide, the hydrogen sulfide with a stream of nitrogen, and the mercuric sulfide filtrate was adjusted to pH 7.4 with sodium hydroxide. The supernatant was treated in the same way; it was roughly 0.13 M with respect to acetate and the volume was increased from 5 to 7 cc.

Result of Experiments with Lymph and Serum

The results are summarized in Table I. In six out of nine experiments the rat liver slices show a higher oxygen consumption in the lymph taken some time after the burn than in the normal lymph, and this difference is definitely outside the experimental error. In three experiments the increase is not in itself significant, but the fact that the Q_{O_2} in the lymph after the burn is in no case below normal lends further support to the findings in the other experiments. The experiments on Calves 10 and 13 show that the effect increases with time. Whether the effect might diminish later was not determined. Calf 9 was the only animal kept under observation for more than 22 hours and in this case the effect was so small that measurements were discontinued.

The results with serum are a little less consistent but here, too, the tendency is towards a higher oxygen consumption in the serum taken after the burn. The experiment on Calf 13 shows the variations found when livers from different rats were used with the same sera. The effects observed with three livers are very similar and indicate that the variations in the responses found with different animals were not due to differences in the rat livers.

In order to be certain that the increase in oxygen consumption caused by lymph from burned regions in calves was not a species phenomenon but was found in the exudate from burns in other mammals, a dog was anesthetized with nembutal, just as in the case of the calves, and all the lymph from the hind legs was collected by introducing a cannula into a single large vessel at the lower end of the receptaculum chyli. This is an ex-

tremely satisfactory method of preparation and gives a copious flow of lymph when the legs are kept in passive motion by attachment to a motor-

TABLE I
Oxygen Consumption of Rat Liver Slices in Serum and Lymph from Calves before and after Burns

Calf No.	Time after burn	Time oxygen consumption was determined	QO ₂ , lymph		Difference	QO ₂ , serum		Difference
			Normal	After burn		Normal	After burn	
	hrs.		c.mm. per mg. per hr.	c.mm. per mg. per hr.	per cent	c.mm. per mg. per hr.	c.mm. per mg. per hr.	per cent
1	11½-15½	1st hr.	12.5	16.7	+34			
		2nd "	11.9	16.6	+40			
2	14-18	1st "	15.7	19.7	+25			
		2nd "	14.9	20.0	+34			
5	18-10	1st "	10.8	11.2	+4	9.6	13.1	+36
		2nd "	10.8	10.8	0			
	10	1st "				12.2	12.9	+6
		2nd "				8.7	12.9	+48
	10	1st "				12.2	13.6	+11
		2nd "						
6	13-17	1st "	10.2	12.7	+25			
		2nd "	8.2	10.8	+31	8.5	10.2	+20
	13-17*	1st "	(10.0)	(10.5)	(+5)			
		2nd "	(8.2)	(9.2)	(+12)			
7	11½-15½	1st "	13.5	13.9	+3			
9	6-8	1st "	10.7	11.7	+9			
		1st "	13.9	14.9	+7	14.3	13.9	-3
10	½	1st "						
		1st "	9.1	{ 9.4	+3			
	8-12	1st "		{ 11.1	+22			
		1st "		{ 10.1	+16			
	4-6	1st "	8.7	{ 12.3	+41			
		1st "				13.1	13.5	+3
12	12	1st "						
		1st "		{ 11.4	+14			
	15-17	1st "	10.0	{ 11.9	+19			
		1st "		{ 10.0	+15			
13	4-6	1st "		{ 11.0	+26			
		1st "	8.7	{ 12.1	+41			
	6-8	1st "				8.9	9.9	+11
		1st "				11.6	12.3	+6
	17-21	1st "				9.7	10.8	+11
		1st "						
	22	1st "						
		1st "						

Braces indicate that the determinations were made simultaneously on the same liver. This is also indicated for the determinations on lymph and serum when the results are on the same line.

* The second experiment was done on the same samples as the first but both were diluted 1:4 with Ringer-phosphate solution.

driven crank just as was done in the experiments upon calves. After a sufficient amount of lymph was collected, the animal was burned under

anesthesia by immersion of the legs and tail in water at 100° for 1 minute. This amount of time results in a more severe burn in the dog than does the longer period with the calves. The dog survived for 4 hours and the lymph collected from the burned region during the last hour was even more potent than calf lymph obtained in a similar way in augmenting oxygen utilization. (Q_{O_2} , c.mm. per mg. per hour, normal lymph 6.6, lymph after burning 10.1, increase 53 per cent.)

In a control experiment on a calf kept under nembutal anesthesia for 24 hours and treated exactly as were the other animals except that the burn was omitted, the following values for the Q_{O_2} were found: in lymph taken during the first 4 hours 9.7 c.mm. per mg. per hour, in lymph taken during the last 4 hours 9.8 c.mm. per mg. per hour.

Another possible source of error was realized after the experiment on Calf 11. Blood pressure was measured intermittently during the experiment from the carotid artery which was connected with a manometer through a tube filled with 10 per cent sodium citrate. Citrate added to the medium is known to cause a slight increase in the oxygen consumption of rat liver slices (8). In the last two experiments heparin was therefore used to prevent coagulation in the tube. The results show that citrate which might inadvertently have entered the circulation was not responsible for the effect. A direct experiment on the serum before and after the injection of a dose of citrate much larger than the amount which could possibly have leaked back into the animal during the course of the experiment was negative.

The average Q_{O_2} in normal lymph is 11.3 c.mm. per mg. per hour; in normal serum it is 11.4 c.mm. per mg. per hour. This is higher than the Q_{O_2} usually found when Ringer-phosphate solution is used as the medium; an average Q_{O_2} of 9.5 c.mm. per mg. per hour was found by the same experimenter in a similar series (6). It is somewhat lower than the results reported by Friend and Hastings (4) who found an average of 12.5 c.mm. per mg. per hour for rat liver slices in normal human serum. Dickens and Šimer (9) found less of a difference between serum and Ringer's solution. Canzanelli, Rogers, Dwyer, and Rapport (10) found that the Q_{O_2} for guinea pig liver in horse serum was more than twice as great as in "NaCl-Sørensen- PO_4 " solution. These results are not quite comparable with ours, since the species and the medium were not the same.

DISCUSSION

Many different substances are known to increase the Q_{O_2} of rat liver slices in Ringer-phosphate solution when no other substrate is present. Some experiments have been reported on the effect of the addition of other substrates when there is already an ample supply of glucose. Often the effect is more pronounced when the liver of a fasted rat is used (see *e.g.*

(8, 11, 12)). Since the addition of glucose to the medium in which normal liver slices are respiring has very little effect on the oxygen consumption, it can probably be assumed that substrates which increase the metabolism of normal liver slices would have the same effect whether or not glucose is present.

As already pointed out, normal serum and lymph contain some substances which increase the oxygen consumption of liver slices. Canzanelli *et al.* (10) found that in normal serum most, if not all, of the increase was due to substances present in the ultrafiltrate. Table II shows that the same is true for both normal lymph and lymph after burning, which means

TABLE II
QO₂ of Rat Liver Slices in Lymph after Different Treatments

Calf No.	Time after burn			QO ₂		
				Normal lymph	Lymph after burn	Ringer's solution
	hrs.			c mm. per mg per hr.	c mm. per mg per hr.	c mm. per mg per hr.
11	8-14	Ultrafiltrate from lymph		13.2	19.4	
13	17-21	Ultrafiltrate from lymph		9.6	10.9	5.8
		Ultrafiltrate after ether extraction	Extract	{ 8.5	8.5	6.4
			Residue	{ 7.5	8.8	
		Ultrafiltrate after pptn. with mercuric acetate	Ppt.	{ 9.2	11.1	8.3
			Supernatant	{ 11.2	13.9	11.1
		Whole lymph, liver from fasted rat	No glucose	{ 7.2	8.8	8.1
			With glucose	{ 7.1	9.9	8.2

Each set of results enclosed in a brace was obtained simultaneously on liver slices from a single animal.

that not only the substance from normal lymph but also that present in the lymph from the burned leg is ultrafiltrable.

In order to decide whether the increase observed after burning is due to an increase in the amount of substances already present or to the release of some substances not normally present in the circulation, it will be necessary to effect at least partial separation of these substances. The very extensive literature on tissue metabolism offers very little help in devising methods of separation, because such a large variety of compounds has been found to influence the metabolism. Therefore, in a first attempt to concentrate the active material, reagents which would remove a large number of organic compounds were used. The scarcity of available material seriously limited the choice of methods. Furthermore it was de-

sirable to use reagents that could be removed in such a way that both the material isolated from the ultrafiltrate and the residue could be tested for their effect on liver slices.

Ether extraction was first tried. The procedure is given in the experimental part and the results in Table II. Both this and the following are single experiments and, therefore, should not be given too much weight. Apparently most of the substances responsible for the difference between oxygen consumption in normal lymph and in Ringer-phosphate solution are extracted by ether from acid solution. The effect of the ether extract from lymph after burning is the same as that of the ether extract from normal lymph. The difference between the oxygen consumption in ultrafiltrate from lymph before and after burning is still present in the residues after the ether-soluble substances have been removed; *i.e.*, the active material released after burning cannot be extracted with ether under these conditions, while most of the active material normally present can be extracted.

Mercuric acetate was used next because of the large variety of biological materials precipitated by mercury. The results, recorded in Table II, are not as clear cut as those in the previous experiment and might indicate the presence of several active substances. The Ringer-phosphate solution in this experiment was subjected to the same treatment as the ultrafiltrate and the high values for Q_{O_2} in the supernatant fluids are probably due to the high concentration of acetate (0.13 M) (12).

The experiment in which the liver from a fasted rat with and without added glucose is employed points to the presence of an activator rather than a substrate. Even without added glucose the concentration at the end of the experiment was 0.005 M glucose; with addition it was 0.015 M. It seems unlikely that a substrate should have more of an effect when the concentration of glucose is increased.

The last experiment incidentally showed that the active material is quite stable. The lymph was sterilized by filtration through a Seitz filter shortly after it was collected and was then stored in an ordinary refrigerator. After 1 week, the time elapsed between this experiment and the first (see Table I), there was no detectable decrease in the activity. The first experiment showed 41 per cent increase, the last, with glucose, 39 per cent increase.

The finding of a substance in lymph from severe burns which increases oxygen consumption of rat liver slices, together with the few indications we have gained as to the nature of the compounds involved, tempts one toward attractive series of generalizations relative to inflammation. It is, however, our feeling that while the facts we have reported are quite definite other experiments should be done before they are given general

biological significance. Experiments on the effect of lymph from burned areas on muscle metabolism are now being undertaken.

SUMMARY

Lymph was collected from the legs of calves under nembutal anesthesia before and after burning by immersion in boiling water for 3 to 3½ minutes as previously described.

The oxygen consumption of rat liver slices was measured with the lymph as the medium. It was found that lymph coming from a burned area contained some substance or substances which caused an increase in the Q_{O_2} . The effect increased with time. The Q_{O_2} in lymph after burning was as much as 41 per cent higher than that in normal lymph.

Less consistent results were obtained with serum collected before and after burning, but here too the tendency was towards a higher oxygen consumption in the serum after burning.

A similar experiment on the lymph from a dog showed even more of an increase in the Q_{O_2} than did the experiments on calves.

The material which caused the increase in oxygen consumption was found to be present in the ultrafiltrate from the lymph after burning.

A few attempts to concentrate the active material are recorded.

It is a pleasure to express our thanks to Dr. Cecil K. Drinker for making possible this work and for extending the hospitality of his laboratory.

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THE MANNER OF INACTIVATION OF THIAMINE BY FISH TISSUE

BY L. O. KRAMPITZ AND D. W. WOOLLEY*

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

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After the discovery by Green, Carlson, and Evans (1) that a disease of foxes caused by feeding raw carp could be prevented or cured by thiamine, and the demonstration in this laboratory (2) and elsewhere (3) that carp contained a heat-labile substance which would inactivate thiamine *in vitro*, it was of interest to determine in what manner this destruction was accomplished. It was desired to learn in particular whether thiamine was inactivated by combination with a specific protein in a manner similar to the inactivation of biotin by antibiotin (avidin) (4, 5), or whether it was destroyed by enzymic action. The combination hypothesis was not easy to disprove, for, although the reaction was not instantaneous (2, 3), it was conceivable that combination might proceed slowly. Several characteristics of enzymic action other than finite speed (such as heat inactivation) could also be characteristics of the stoichiometric combination reaction. Nevertheless, Sealock *et al.* (6) have presented evidence based on kinetic studies in support of the enzyme hypothesis.

The enzymic nature of the inactivation of thiamine by carp tissue was proved by demonstration of fragments produced from thiamine by the action of the tissue. These products have been identified as 4-methyl-5-hydroxyethylthiazole and 2-methyl-4-amino-5-hydroxymethylpyrimidine. Thus the over-all reaction was a hydrolytic cleavage of the vitamin between the pyrimidine and thiazole rings.

The demonstration of the products of reaction was first accomplished microbiologically. Although NaCl extracts of carp viscera completely destroyed thiamine, as measured by animal assay or by chemical determination (1-3), they did not cause any loss of potency of thiamine when the assay was made with *Mucor ramannianus*, a mold which required thiamine or merely its thiazole portion¹ for growth (7). Furthermore, dialysates of a reaction mixture of thiamine and fish extract, in which all thiamine had

* With the technical assistance of A. G. C. White.

¹ Throughout this paper, the "thiazole portion" of thiamine refers to 4-methyl-5-hydroxyethylthiazole, and the "pyrimidine portion" refers to 2-methyl-4-amino-5-hydroxymethylpyrimidine. We wish to thank Dr. J. C. Keresztesy and Dr. R. T. Major, of Merck and Company, Inc., for gifts of several thiazoles and pyrimidines used in this investigation.

been destroyed, were as potent as the original thiamine when tested with this mold. These facts indicated that 4-methyl-5-hydroxyethylthiazole was a product of the reaction. This was confirmed by isolation and characterization of the pure thiazole from the reaction mixture. When thiamine-free reaction mixtures obtained from carp extract and thiamine were tested with *Endomyces vernalis*, a yeast which required thiamine or merely its pyrimidine portion for growth (2, 8), it was found that only a small fraction of the activity of the original thiamine remained. However, when similar reaction mixtures obtained by the use of carp tissue, rather than carp extract, were assayed by this method, all the activity of the original vitamin was found present. From such reaction mixtures, 2-methyl-4-amino-5-hydroxymethylpyrimidine was isolated and characterized.

The difference in action of carp tissue and of NaCl extracts of the tissue, referred to above, indicated that a series of reactions may have been involved. The primary reaction would be the liberation of the thiazole portion of the vitamin and the formation of a pyrimidine derivative which was only slightly active for *Endomyces*. Then a substance present in the tissue, but almost absent from the NaCl extract, would convert this intermediate to 2-methyl-4-amino-5-hydroxymethylpyrimidine. There were alternative explanations of the observed difference, but support was lent to the present hypothesis by the observations that various batches of extract of equal thiamine-destroying potency produced varying amounts of the pyrimidine active for *Endomyces*, and that thiamine-free mixtures of NaCl extracts and thiamine slowly increased in potency for *Endomyces* when they stood for long periods. Finally, treatment of the reaction mixture of extract and vitamin with alkali brought about full restoration of activity for *Endomyces*. 4-Methyl-5-hydroxyethylthiazole was isolated from the reaction mixture of carp tissue and thiamine, as well as from the reaction mixture obtained with the extract; hence, the free thiazole was produced in the primary reaction.

The thiamine-destroying activity of NaCl extracts of carp tissue resided in two fractions, one dialyzable and the other non-dialyzable. Either fraction alone had some slight activity against thiamine, but the two together were necessary for full activity. Certain other characteristics of the reaction supplementing those recently reported by Sealock *et al.* (6) will be described in the experimental part.

EXPERIMENTAL

Chemical Method—A series of centrifuge tubes, each containing 50 γ of thiamine, enough water to make 20 cc., and a suitable dilution of suspensions or extracts of carp viscera, was incubated for 1 hour at 25°.

The solutions were then acidified to pH 2 to 3 and treated with Super Filtrol. Thiamine was then determined in the Super Filtrol adsorbates, according to Emmett *et al.* (9). The latter method was modified to include extraction of the colored derivative with xylene. Subtraction of the number of micrograms of thiamine found from 50 gave the amount destroyed by the preparation. A curve was constructed which related the thiamine destroyed to the quantity of fish preparation used, and the amount of preparation which destroyed 25 γ of thiamine was determined. This amount was said to contain 1 unit of activity.

Endomyces Method—After the reaction of thiamine with the fish preparation, the material was assayed according to directions given previously (2, 8).

Mucor Method—The assay was conducted in the same way as with *Endomyces* (2, 8), except that the inoculum was a suspension of spores washed from a culture of *Mucor ramannianus* grown on an agar slant. The culture for the inoculum was grown for a month on 5 cc. of the basal medium solidified with agar. 5 cc. of spore suspension were obtained from 5 cc. of solid medium. The spores were washed three times with sterile water. The inoculated flasks were incubated for 10 days at 25° and then the mycelium in each was filtered off, washed, dried, and weighed. The weights so obtained from flasks containing known amounts of thiamine were used to construct a standard curve, and the values of the unknowns were estimated in the usual manner.

Unless otherwise stated, all determinations of thiamine were performed according to the chemical method.

Preparation of Carp Extract—Since it was shown previously (2, 3, 6) that most of the thiamine-destroying activity was in the viscera, this source was used in preference to the whole fish. The viscera were removed immediately after the fish were killed and were used at once. 1 gm. of viscera destroyed about 1 mg. of thiamine in 1 hour, but did not destroy appreciably more during 24 hours of incubation.

The viscera were ground with 2 volumes of a 10 per cent solution of NaCl in a Waring blender and the suspension was allowed to stand overnight at 4°. It was then filtered through a pad of Filter-Cel. This operation was slow and hence was carried out in a cold room. The clear extract was then assayed and stored at 4°. The preparation was stable for at least a month. The thiamine-destroying potency of graded amounts of such an extract is shown in Table I.

Effect of Temperature, pH, and Time of Incubation—The amount of thiamine destroyed was increased as the temperature was raised from 0° to 37°, but the temperature coefficient over the range was small (Table II). Likewise, the amount of thiamine destroyed was influenced but little by

changes in pH over the range 1 to 8 (Table II). The amount destroyed was increased slightly as the pH was raised. It was shown that part of the lowered activity at pH 1 was due to the instability of the enzyme at that pH. The pH range above 8 could not be investigated because of the instability of thiamine in alkaline solution.

The amount of thiamine destroyed by a given amount of carp extract increased regularly with the time of reaction for approximately the first

TABLE I

Destruction of Thiamine by Varying Amounts of Carp Extract

Each reaction mixture contained originally 50 γ of thiamine.

Carp extract	Thiamine destroyed
	γ
0	0
0.05	6
0.1	16
0.3	23
0.5	32
1.0	50

TABLE II

Effect of Temperature, pH, and Time on Destruction of Thiamine by Carp Extract

Each reaction mixture contained originally 50 γ of thiamine.

Effect of temperature		Effect of pH		Rate of destruction	
Temperature	Thiamine destroyed	pH	Thiamine destroyed	Time of reaction	Thiamine destroyed
$^{\circ}\text{C.}$	γ		γ	<i>min.</i>	γ
0	21	1	12	0	12
25	25	4.4	23	5	24
30	27	5	27	15	30
37	33	6	38	30	32
		7	47	60	32
		8	50	120	32

quarter hour. Following this, no more of the vitamin was destroyed. Data to illustrate this point are shown in Table II. The same cessation of the reaction after about the 1st hour was likewise observed when suspensions of carp viscera were used in place of the extract. This phenomenon was not explainable by the inhibitory effect of the products of reaction, for although these products did inhibit the reaction the effect was not marked. Thus, when an extract which would destroy 46 γ of thiamine in 1 hour was incubated with 400 γ of either of the reaction products (*i.e.*,

2-methyl-4-amino-5-ethoxymethylpyrimidine and 4-methyl-5-hydroxyethylthiazole) or the two together for 1 hour, it was found subsequently to destroy 41 γ of thiamine in 1 hour.

Demonstration of Enzymic Nature of Reaction by Recognition of Reaction Products—The formation of a dialyzable fragment of the thiamine molecule during the reaction was demonstrated in the following manner. 10 cc. of carp extract were allowed to react with 500 γ of thiamine for 1 hour. The absence of thiamine in the reaction mixture was demonstrated by the chemical assay, but no loss of thiamine was detected by the *Mucor* test. The reaction mixture was then dialyzed against 100 cc. of water for 20 hours.

TABLE III
Thiamine-Destroying Potency of Fractions of Carp Extract

Carp extract	Amount used*	Thiamine destroyed†
	cc.	γ
Extract	1	50
	0.5	32
Non-dialyzable portion of extract	2.0	22
	1.0	18
	0.5	12
Dialysate of extract	2.0	6
	1.0	5
	0.5	3
Dialysate + non-dialyzable portion of extract	2.0 + 2.0	47
	1.0 + 1.0	46
	0.5 + 0.5	30
Boiled extract	1.0	2
Non-dialyzable portion + boiled extract	1.0 + 1.0	42
	1.0 + 0.5	31

* The volumes of the non-dialyzable portion and the dialysate were adjusted to correspond with the original volume of the carp extract.

† Each reaction mixture contained originally 50 γ of thiamine.

The dialysate was then analyzed by the *Mucor* method, and found to contain 90 per cent of the thiamine activity started with. It was subsequently shown by isolation and characterization that the *Mucor*-active substance formed was 4-methyl-5-hydroxyethylthiazole (see below).

Partition of Enzyme by Dialysis—Carp extract, which would destroy 64 γ of thiamine per cc. of extract under the test conditions, was dialyzed against eight changes of distilled water, and the dialyzable and non-dialyzable fractions were tested for thiamine-destroying potency. The results are shown in Table III. It can be seen that either fraction alone had slight activity against thiamine, but that, when the two were combined, the original activity of the extract was regained. Exhaustive dialysis of carp

extracts against running water for 48 hours did not completely inactivate them. Residual activity amounting to about 10 per cent of the original always remained after exhaustive dialysis.

The dialyzable component was heat-stable, while the non-dialyzable part was heat-labile. Thus, when heated dialysate or heated carp extract was added to the non-dialyzable fraction, activity was regained, while, when a heated non-dialyzable fraction was added to the dialysate, no activity was generated. The heating in these experiments was done at 100° for 15 minutes.

Isolation and Identification of Products of Reaction. 4-Methyl-5-hydroxyethylthiazole—The isolation of this component of the reaction products was accomplished with the aid of the *Mucor* assay which was used to guide the operations. 2.5 liters of carp extract and 1 gm. of thiamine were mixed and allowed to stand overnight at 4°. The pH was maintained above 7 with NaHCO₃. The solution, which was then free of thiamine, was concentrated under reduced pressure until NaCl began to crystallize, and was then treated with 4 volumes of alcohol. The precipitate was filtered off and washed with alcohol, and the filtrate was concentrated under reduced pressure to a sirup. This sirup was taken up in a small amount of alcohol, and the insoluble matter was removed. The extract was freed of alcohol and taken up in a small amount of water, made alkaline with ammonia, and extracted three times with chloroform. The chloroform was removed from the extract under reduced pressure and the pale yellow liquid which remained was dissolved in a small amount of absolute ethanol and treated with excess alcoholic picric acid. The crystals which formed were filtered off and recrystallized, m.p.² 156–158°. Synthetic 4-methyl-5-hydroxyethylthiazole picrate in the same bath melted at 156–158°. The yield was 930 mg. (82 per cent of theory).

The free base was regenerated and the chloroplatinate was prepared from it, m.p. 172–175°. Authentic 4-methyl-5-hydroxyethylthiazole chloroplatinate in the same bath melted at 172–175°.

$C_{13}H_{20}O_2N_2S_2PtCl_6$.	Calculated.	C 20.7, H 2.87, Pt 28.0
	Found.	" 20.7, " 2.99, " 29.0

The flavianate was prepared and found to melt at 188–191°, with decomposition. An authentic sample melted at 190–192°, with decomposition, in the same bath. Mixture of each of the three derivatives with the appropriate authentic specimen resulted in no depression of the melting points.

2-Methyl-4-amino-5-hydroxymethylpyrimidine—The *Endomyces* method was used throughout the following fractionation scheme to guide the opera-

² All melting points recorded in this paper were uncorrected.

tions. For reasons which will appear below, a suspension of carp viscera was used in preference to carp extract.

900 gm. of carp viscera and 400 cc. of water were thoroughly mixed in a Waring blender, and to the mixture was added 1 gm. of thiamine. The mixture was allowed to stand overnight at 4°, and was then found to be free of thiamine. 97 per cent of the original thiamine activity for *Endomyces* was present, however. 5 liters of alcohol were added, and the precipitate was filtered off and washed with alcohol. The filtrate and washings were concentrated under reduced pressure to a sirup which was dissolved in 1 liter of water, made to pH 9 with barium hydroxide, and filtered. This alkaline solution was extracted continuously with ether for 24 hours. 4-Methyl-5-hydroxyethylthiazole picrate, m.p. 156–158°, was isolated from the ether extract. The residual aqueous solution was concentrated to 400 cc. under reduced pressure and mixed with 5 per cent of its volume of sulfuric acid, and then with an excess of mercury sulfate in 5 per cent sulfuric acid. The precipitate was filtered off, and the filtrate was freed of mercury with H₂S. The HgS filtrate was extracted ten times with 300 cc. portions of butanol. The residual aqueous solution was made to pH 9 with barium hydroxide, and the barium sulfate was filtered off. The filtrate was then extracted ten times with butanol. The alkaline butanol extracts were concentrated under reduced pressure to 30 cc., filtered, and concentrated to dryness. The residue was dissolved in 20 cc. of water, and the solution was extracted continuously with chloroform for 24 hours. The extract was freed of solvent under reduced pressure, dissolved in water, and decolorized with 50 mg. of norit. The solution so obtained was concentrated under reduced pressure to dryness. This residue was a glass which slowly crystallized. The crystals melted at 195°. Andersag and Westphal (10) reported the melting point of 2-methyl-4-amino-5-hydroxymethylpyrimidine as 194°. The material was 2.3 times as active as an equal weight of thiamine in the *Endomyces* test and hence was practically pure by this criterion, since the molecular weight of thiamine is approximately 2.4 times that of the pyrimidine. The yield was 160 mg. (41 per cent of theory).

The hydrochloride was prepared in alcohol and precipitated with ether.

C ₈ H ₁₀ ON ₂ Cl.	Calculated.	C 41.1, H 5.71, N 24.0
	Found.	" 41.4, " 5.55, " 24.0

The chloroplatinate was prepared from the hydrochloride.

C₁₂H₂₀O₂N₂PtCl₆. Calculated, C 20.9, H 2.9; found, C 21.3, H 3.1

Evidence for Stepwise Course of Reaction—Evidence for the formation of a compound inactive for *Endomyces*, but which was converted into 2-methyl-4-amino-5-hydroxymethylpyrimidine during the course of the

reaction, was obtained in the following manner. Although suspensions of carp tissue reacted with thiamine to produce a substance (identified above) with no detectable loss of thiamine potency for *Endomyces*, NaCl extracts of the tissue caused marked loss of thiamine potency as measured by *Endomyces*. Thus in three separate runs suspensions of carp viscera destroyed all of the thiamine added, but the reaction mixtures exhibited 97, 95, and 85 per cent of the original thiamine potency when tested with *Endomyces*. On the other hand, NaCl extracts of carp viscera reacted with thiamine to leave only a fraction (varying from 5 to 30 per cent) of the original activity for the yeast.

The following experiments indicated that a biologically inactive derivative of the pyrimidine was formed during the destruction of thiamine with carp extract. Long incubation of the thiamine-free mixture of extract and vitamin resulted in the slow generation of thiamine activity for *Endomyces*.

TABLE IV

Rate of Appearance of Thiamine Activity for Endomyces in Thiamine-Free Mixtures of Carp Extract and Thiamine

Time of incubation	Thiamine activity for <i>Endomyces</i>
<i>hrs.</i>	<i>per cent of original thiamine</i>
1	25
6	28
24	39
192	70

This fact was illustrated by the data in Table IV. In this experiment, 10 cc. of extract and 100 γ of thiamine were incubated at 4°, and aliquots were analyzed at the stated intervals. Furthermore, digestion of the reaction mixture with 1 N NaOH regenerated all of the original thiamine potency for this organism. The fish tissue probably contained an enzyme (which was nearly absent from the extract) which liberated the pyrimidine from this intermediate. The slow increase in potency observed with the extract would then be due to a trace of this second enzyme in the extract. The varying potency of extracts referred to in the previous paragraph would likewise be explained by such a hypothesis.

SUMMARY

The destruction of thiamine by carp tissue has been shown to be an enzymic reaction by the demonstration of the products of cleavage of the thiamine molecule. The reaction was but little affected by changes in pH in the range 1 to 8 and by changes in temperature from 0° to 37°. The enzyme has been obtained in solution in 10 per cent NaCl in good yield,

and has been shown to consist of a heat-labile, non-dialyzable part and a heat-stable, dialyzable component. The over-all reaction in carp tissue suspensions was the rupture of the vitamin by the addition of water to yield 4-methyl-5-hydroxyethylthiazole and 2-methyl-4-amino-5-hydroxymethylpyrimidine. These substances were isolated and characterized. Evidence was presented to show that an intermediate compound was formed which was transformed into the above pyrimidine in tissue suspensions, but only very slowly in NaCl extracts.

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A METHOD FOR THE DETERMINATION OF COPROPORPHYRIN IN URINE

By HAROLD L. MASON

(From the Division of Biochemistry, The Mayo Foundation, Rochester, Minnesota)

AND SAMUEL NESBITT

(From the Department of Medicine, Yale University School of Medicine, New Haven)

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Urinary coproporphyrin has been estimated by a variety of methods in which fluorometric, colorimetric, and spectroscopic procedures have been used (1-4, 6-11). The fluorometric procedure is very sensitive and permits detection of minute amounts of porphyrin. Since a procedure adapted to the use of a modern fluorophotometer has not been described, we wish to present the details of a method which has been in use for several years in these laboratories. Most of the procedures used are adaptations of those described by Brugsch (1), Fikentscher (4), and Fischer (5).

In the following discussion it is assumed that coproporphyrin is the only porphyrin present in urine which is extracted with ether in the presence of acetic acid and in turn is removed completely from the ether by a few extractions with 5 per cent hydrochloric acid. This assumption undoubtedly is justified in the great majority of instances but possible exceptions should be kept in mind. No provision for hydrolysis of possible conjugates (8) of coproporphyrin has been made in the routine procedure. This point should receive further investigation.

A Pfaltz and Bauer fluorophotometer, model A, was used for most of this work. A Coleman, model 12, fluorophotometer also was used by one of us (S. N.). The results given for Patients 4 to 11 were obtained with this instrument. In both instruments a 100 watt General Electric H-4 type mercury lamp is used for the exciting light. The incident beam passes through the solution contained in a $1 \times 4 \times 4.5$ cm. cuvette in the case of the Pfaltz and Bauer instrument and through a $\frac{3}{4}$ inch (1.9 cm.) test-tube in the case of the Coleman instrument. The intensity of the fluorescent light which is emitted at right angles to the incident beam is measured by a photocell placed appropriately. For this determination the incident light was filtered with a Corning Filter 511 of 1 mm. thickness. The maximal transmission of this filter is about $410 m\mu$. The fluorescent light passed through a red filter which cut off sharply radiation below $578 m\mu$ to filter out scattered light. In order to increase the sensitivity a mirror was placed behind the cuvette or the test-tube so that fluorescent light which otherwise would have been lost was reflected back through the solution of porphyrin

onto the photocell. A mask was used over the end of the cuvette which received the incident light and also over the side facing the photocell. These masks covered the upper part of the cuvette so that the meniscus was not illuminated and did not contribute to the fluorescence. The cuvette then could be filled without attention to the exact volume as long as the meniscus was above the openings in the masks. Without the masks it was necessary to use always an exact volume of the porphyrin solution. These precautions were unnecessary with the test-tube of the Coleman instrument.

Method

Collection of Urine—Urine is collected for 24 hours in dark bottles which contain toluene as a preservative. It may be kept in a refrigerator for several days without appreciable loss of porphyrin.

*Standard*¹—The stock standard is a solution of coproporphyrin in 5 per cent hydrochloric acid which contains 10 γ per ml. The working standard is obtained by dilution of 0.3 ml. of the stock solution to 100 ml. with 5 per cent hydrochloric acid. Both standard solutions should be protected from light.

Coproporphyrin for the standard was isolated from the urine of patients suffering from hepatic disease and of those suffering from acute porphyria. It was crystallized as the methyl ester (6, 12). For preparation of the stock standard 1 to 3 mg. of the ester was weighed on a micro balance and dissolved in 10 to 20 ml. of 25 per cent hydrochloric acid. When all the ester was dissolved, the solution was diluted with water to make the concentration of acid 5 per cent. It then was diluted with 5 per cent hydrochloric acid to the volume required to make the concentration of coproporphyrin 10 γ per ml.; 1.086 mg. of the tetramethyl ester are equivalent to 1 mg. of coproporphyrin.

Procedure

A 25 ml. aliquot of urine is acidified with 10 ml. of glacial acetic acid and then thoroughly shaken with 35 ml. of ethyl ether in a 150 ml. separatory funnel. If the amount of porphyrin in the total quantity of urine is known to be greater than 100 γ , an aliquot which contains not more than 5 γ is used and this is diluted to 25 ml. If an emulsion forms, it can be broken usually by addition of a few drops more of glacial acetic acid. The two layers are separated and the urine is extracted twice more with 35 ml.

¹ Ewing and Cornbleet (3) have used a commercial preparation of hematoporphyrin hydrochloride (photodyne) as a standard for the photometric determination of coproporphyrin. Presumably hematoporphyrin could also be used as a fluorometric standard once the relation between the fluorescence of hematoporphyrin and coproporphyrin was established.

portions of ether. The ether extracts are combined and washed three times with 25 ml. portions of water. The combined water washings are washed back once with 25 ml. of ether which are added to the other ether extracts. The ether solution, which now contains all of the coproporphyrin, is extracted with 5 ml. portions of 5 per cent hydrochloric acid. Ordinarily three extractions are sufficient but if the amount of porphyrin has been underestimated more extractions may be necessary. Consequently a fourth extract is always made and examined in the incident beam of the fluorophotometer with Filter 511. If a red fluorescence is observed, the extraction is continued until a subsequent extract does not exhibit any fluorescence. All of the 5 per cent hydrochloric acid extracts which contain porphyrin are combined and the volume is measured, or the solution may be diluted to a convenient volume.

The ether which is dissolved in the extracts does not affect the determination but since it increases the volume the final volume must be measured. The dilution of the acid which results is not sufficient to affect appreciably the intensity of fluorescence of the porphyrin. Although the intensity of fluorescence of a porphyrin depends on the acidity of the solution, so that it is important to keep the concentration of acid constant for standard and unknown, the concentration of 5 per cent hydrochloric acid is sufficiently large that small deviations from this concentration do not result in measurable differences in the intensity of fluorescence.

With the standard solution in the cuvette the intensity of the incident light is adjusted by means of the iris diaphragm until the galvanometer reading is almost at the maximal position on the scale (20 cm. with the instrument used). Then the extract is substituted for the standard and the reading taken. The extract is diluted, if necessary, with 5 per cent hydrochloric acid until the reading is less than that of the standard. In order to determine whether there are substances present which interfere with the fluorescence of the porphyrin, a portion of the solution which gave a satisfactory reading is diluted further with an equal volume of the 5 per cent hydrochloric acid and a reading is taken with this dilution. If the two readings are not proportional, there has been interference with the fluorescence and it is necessary to purify the porphyrin as described subsequently. A blank reading, taken with the 5 per cent hydrochloric acid in the cuvette, is subtracted from all readings.

Calculation—The relation of the reading and concentration of the unknown to the reading and concentration of the standard is that of a simple proportion.

If the 5 per cent hydrochloric acid solution contains more than 5 γ of porphyrin, the urine residue should be examined further for completeness of extraction. It is extracted a fourth time with 35 ml. of ether and the

ether is extracted with 5 ml. of 5 per cent hydrochloric acid. The hydrochloric acid extract is examined in the fluorometer. If no red fluorescence is observed, it may be concluded that the first three extracts contain all of the porphyrin. If there is a red fluorescence, the hydrochloric acid extract is neutralized to Congo red with solid sodium acetate and shaken again with the ether from which it was derived. The urine residue also is extracted further until an acid extract of the ether no longer shows a red fluorescence. The combined ether extracts are treated as outlined previously.

With the instruments used as described the readings are directly proportional to the concentration of porphyrin between 0 and 0.05 γ per ml. The sensitivity of the instruments is such that readings within this range of concentration are adequate. If another type of instrument is used, it may be necessary to use greater concentrations of porphyrin. In that case the dilution of interfering substances may not be sufficient. If, on dilution, the readings are not proportional to the concentration, the porphyrin is purified as follows: The 5 per cent hydrochloric acid solution is neutralized to Congo red with solid sodium acetate and, if the volume is not more than 50 ml., it is extracted as described for urine. If the volume is greater, larger amounts of ether may be used. The ether extracts are combined, washed with water (which in turn is washed back with ether), and then with 5 per cent hydrochloric acid. The acid solution is now usually sufficiently free from interfering substances so that a linear relation between readings and concentration of porphyrin is obtained. If not, the purification procedure is repeated.

Use of light in the region transmitted by the filter used appears to make the method reasonably specific for porphyrin. This conclusion is based on the observation that ultraviolet radiation of shorter wave-length usually excites an intense white fluorescence in urine. This fluorescence masks that of porphyrin unless relatively large amounts of porphyrin are present. Some of the material which gives the white fluorescence is carried into the extract which contains the porphyrin and interferes with the determination. When the usual ultraviolet filter was used, it was observed almost always that readings on progressive dilutions of the hydrochloric acid extract were not proportional until the concentration became so small that the error in the readings became unduly large. The interfering material could be removed by further purification but more than one transfer of the porphyrin to ether and then to hydrochloric acid was necessary. In the present procedure interference from this source has not been observed. The small concentrations of porphyrin also make possible sufficient dilution of the extracts so that interference of unknown substances by absorption of incident or of fluorescent light is largely eliminated.

Comment

Table I contains the data assembled from twenty determinations of urinary coproporphyrin with simultaneous recovery experiments. In all, twenty-eight such determinations were made. These twenty were chosen for presentation to include typical results for normal and pathologic urines and to show the extreme deviations (-14 and $+13$ per cent) from 100 per cent recovery. It is shown also that satisfactory results are obtained when the total excretion of porphyrin varies from 6 to 1245 γ . The average recovery for the twenty-eight experiments was 99.6 per cent. Since the electrical supply for the Pfaltz and Bauer fluorophotometer underwent sudden and uncontrollable changes of voltage, the readings with this instrument were subject to an uncertainty estimated to be approximately 5 per cent, in spite of check readings of the standard taken just before each reading of the unknown.

Individual specimens of urine may present special problems. If the urine contains protein, it may be difficult to break the emulsion. However, the usual procedure was applied successfully to the urine of Patient 13, which contained hemoglobin. The urine of Patient 18 formed very stable emulsions which could be broken only by centrifugation. This urine also contained a bright yellow pigment which was ether-soluble and some of which went from the ether into the 5 per cent hydrochloric acid extracts. This colored material apparently did not interfere with the determination, since 95 per cent of added coproporphyrin was recovered. The yellow color was eliminated by the procedure described for removal of interfering substances and the recovery of added porphyrin was then 97 per cent. The lower recovery is given in Table I, since it was obtained by the standard procedure. The determination of coproporphyrin was complicated further by the presence of another ether-soluble porphyrin which was removed only slowly from ether by extraction with 5 per cent hydrochloric acid. A weak red fluorescence still was observed in the fifth and sixth hydrochloric acid extracts. It was concluded that this fluorescence did not represent coproporphyrin and only the first three hydrochloric acid extracts were used for the determination. Even so, the presence of this fluorescent material (porphyrin?) resulted in an overestimation of the coproporphyrin. When the porphyrin was returned to ether and then to 5 per cent hydrochloric acid, a value of 1015 instead of 1245 γ was obtained for the total coproporphyrin. Repetition of the entire determination confirmed these values.

The urine of a patient suffering from porphyria (Patient 20) may also present difficulties. Although uroporphyrin, which is characteristic of such urines, is not extracted from water by ether and acetic acid, it has been observed several times that such an ether extract appeared to contain a

TABLE I
Data on Determinations of Urinary Coproporphyrin

Patient No.	Total volume of urine	Aliquot used	Porphyrin			Recovery	Total porphyrin in 24 hrs.	Comment
			Added	Found	Recovered			
	ml.	ml.	γ	γ	γ	per cent	γ	
1	360	15		0.23			5.6	Normal
		15	4.0	4.17	3.94	98		
2	660	25		3.84			101.0	
		25	2.0	5.94	2.10	105		
3	700	10		0.64			45.0	
		10	3.0	3.35	2.71	90		
4	2200	25		0.20			17.8	Normal
		25	0.53	0.72	0.52	98		
5	1600	25		0.34			21.8	"
		25	0.75	1.13	0.79	105		
6	2640	25		0.81			85.5	"
		25	0.52	1.28	0.47	90		
7	1940	25		1.89			147.0	Alcoholic pellagra
		25	0.94	2.95	1.06	113		
8	1490	25		0.41			24.3	Carcinoma of rectum
		25	0.64	0.96	0.55	86		
9	1280	25		0.56			28.9	" " stomach
		25	1.86	2.45	1.89	102		
10	2430	25		1.03			100.0	Pulmonary tuberculosis
		25	2.55	3.55	2.52	99		
11	2380	25		0.78			74.7	Chronic lymphatic leukemia
		25	1.90	2.61	1.83	96		
12	970	15		4.06			264.0	Idiopathic bilirubinemia
		15	3.0	7.25	3.19	106		
13	3200	25		3.16			406.0	Anemia of obscure origin; hemoglobinuria
		25	3.0	6.42	3.26	109		
14	1670	10		3.95			660.0	Anemia
		10	3.0	6.85	2.90	97		
15	1030	10		2.71			279.0	
		10	3.0	5.46	2.75	92		
16	1315	15		1.08			95.0	Obstructive jaundice
		15	3.0	3.85	2.77	92		
17	1425	10		2.02			288.0	" "
		10	3.0	4.95	2.93	98		
18	1665	5		3.74			1245.0	" "
		5	3.0	6.55	2.81	94		
19	2120	15		1.82			258.0	Cirrhosis of liver
		15	3.0	4.59	2.77	92		
20	560	5		2.06			230.0	Porphyria
		5	3.0	4.99	2.93	98		

porphyrin which was extracted slowly from the ether with 5 per cent hydrochloric acid and then would not return to ether after neutralization to Congo red with sodium acetate. Possibly the ether extracted some porphobilinogen from the urine and this was removed slowly and converted to uroporphyrin by the 5 per cent hydrochloric acid. Whatever the explanation, return of the coproporphyrin to ether and then to 5 per cent hydrochloric acid again served to eliminate this source of interference.

It is recognized that satisfactory recovery of a substance added to a biologic material such as urine by no means guarantees that the determination of the amount of this substance originally present in that material is accurate. The simple additive effect on the physical measurement of some other related or unrelated substance would not be detected in the recovery of added substance. The two instances in the preceding discussion (Patients 18 and 20) illustrate this situation. The method outlined, therefore, should not be used in a purely routine manner but each step in the procedure should be watched critically for evidence of interference by other substances.

SUMMARY

A method for the fluorometric determination of coproporphyrin in urine has been described. Two makes of fluorophotometers have been used and satisfactory recoveries of added coproporphyrin have been obtained.

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URINARY EXCRETION PRODUCTS OF ATABRINE

By JOHN V. SCUDI AND VIOLA C. JELINEK

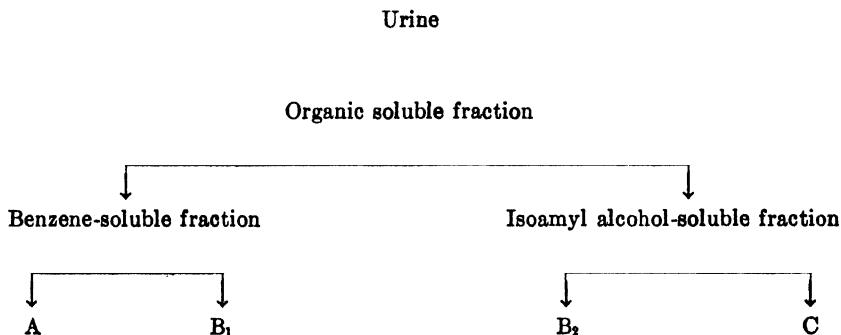
(From the Research Laboratories of Merck and Company, Inc., and the Merck Institute for Therapeutic Research, Rahway, New Jersey)

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Numerous workers have reported that the administration of atabrine imparts an intense yellow color and fluorescence to the urine, and, in general, this appearance of the urine has been attributed to the presence of unchanged atabrine in the specimen. Our experiments indicate that a number of acridine derivatives appear in the urine following administration of the drug to experimental animals.

EXPERIMENTAL

Pooled samples of urine obtained from groups of dogs receiving 10 mg. of the drug per kilo of body weight per day, 6 days of each week, were fractionated according to the accompanying diagram. The details are as



follows: Add strong sodium hydroxide solution to 10 liters of urine (pH above 9.6). Extract twice with 2.5 liter portions of isoamyl alcohol. The alcoholic extract contains, among other things, four basic acridines, tentatively designated Fractions A, B₁, B₂, and C. (Extract the alkaline urine residue with two additional 2.5 liter portions of isoamyl alcohol, and chromatograph directly for the isolation of Fraction C as described later.)

Centrifuge the organic soluble extract to break emulsions and discard the interfacial solids. Add 20 liters of benzene to the isoamyl alcohol and extract the basic acridines¹ with 1000, 500, and 500 cc. volumes of 0.5 N

¹ Hydrolytic cleavage of the basic side chain of atabrine yields the corresponding neutral acridone. Since neutral acridine fractions observed by us at various stages in this work may have resulted from the manipulative procedures used, we have not investigated this fraction.

hydrochloric acid. Discard the organic phase. Add alkali to the acidic solution (pH above 9.6) and reextract the acridines with two 500 cc. portions of isoamyl alcohol. Discard the water phase. Add 4 liters of benzene to the isoamyl alcohol, and extract the acridines with 200, 100, and 100 cc. portions of 0.5 N hydrochloric acid. Discard the organic phase.

Wash the acidic solution with two 100 cc. portions of diethyl ether, and discard the ether extracts. Add alkali to the aqueous phase to pH 8.4 to 8.8 and extract the aqueous solution three times with 100 cc. portions of benzene. Increase the pH of the aqueous phase beyond 9.6, and extract with a final 100 cc. portion of benzene. The combined extracts constitute the *benzene-soluble fraction*. Extract the residual, strongly alkaline, aqueous phase with four 100 cc. portions of isoamyl alcohol. These extracts, combined, constitute the *isoamyl alcohol-soluble fraction*.

Extract the benzene-soluble fraction with four 100 cc. portions of 10 per cent sodium hydroxide to remove Fraction B₁. The residual benzene solution retains Fraction A.

Dry the isoamyl alcohol-soluble fraction with anhydrous sodium sulfate and allow it to percolate through a column of Brockmann's alumina. Fraction B₂ passes into the collecting receptacle, leaving Fraction C adsorbed. Wash the column with ether and air-dry. Elute Fraction C with dilute hydrochloric acid, or preferably with hot water.

Separations effected with the foregoing procedure indicated that there are at least four organic soluble excretion products eliminated by way of the urinary system in the dog. To obtain further information concerning the nature of these products, each fraction was studied individually as follows:

Fraction A—The residual benzene solution, containing Fraction A, pigments, etc., was dried with sodium sulfate and allowed to percolate through a column of alumina 40 to 50 cm. long and 12 mm. in diameter. The chromatogram was developed with benzene containing 5 volumes per cent of isoamyl alcohol. A strongly adsorbed brown band remained at the top of the column. Below this, there was a yellow-orange-colored band, and Fraction A passed into the collecting receptacle. The elution of Fraction A may be accelerated by using higher concentrations of isoamyl alcohol in the eluent.

The eluate containing Fraction A was extracted with 0.5 N hydrochloric acid. The acidic phase was washed with benzene; the pH of the aqueous phase was raised above 9.0 and the acridine was extracted with small volumes of benzene. The benzene extract was dried over sodium sulfate, filtered, and saturated with dry hydrogen chloride. Atabrine dihydrochloride precipitated. After recrystallization from freshly distilled pyridine

the product melted at the same temperature as atabrine dihydrochloride and did not depress the melting point of an authentic sample. The absorption of solutions of this material over the range of 3000 to 5000 Å. was identical with that of solutions of atabrine.

$C_{23}H_{22}ON_3Cl_3$. Calculated. C 58.39, H 6.82, N 8.89
Found. " 58.48, " 6.59, " 8.82

By means of the Beckman spectrophotometer, solutions of atabrine dihydrochloride were examined in the near ultraviolet and visible regions of the spectrum. Dissolved in 1.250 N hydrochloric acid, or in a citrate buffer at pH 3.00, the product gave the same absorption data (Curve 1,

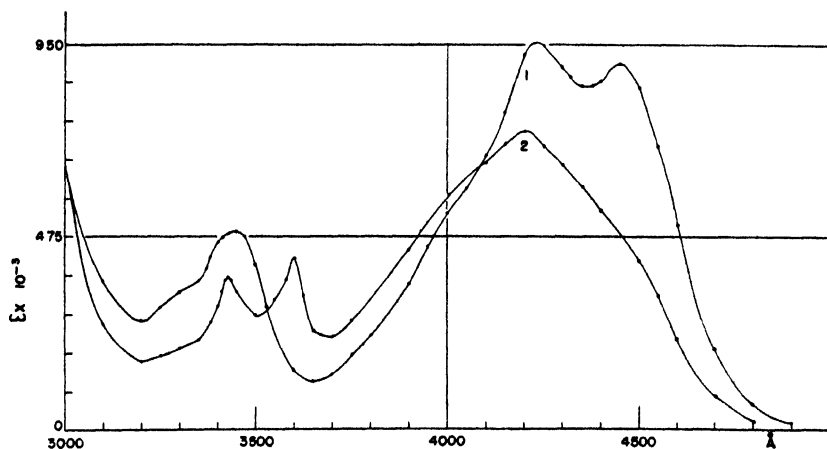
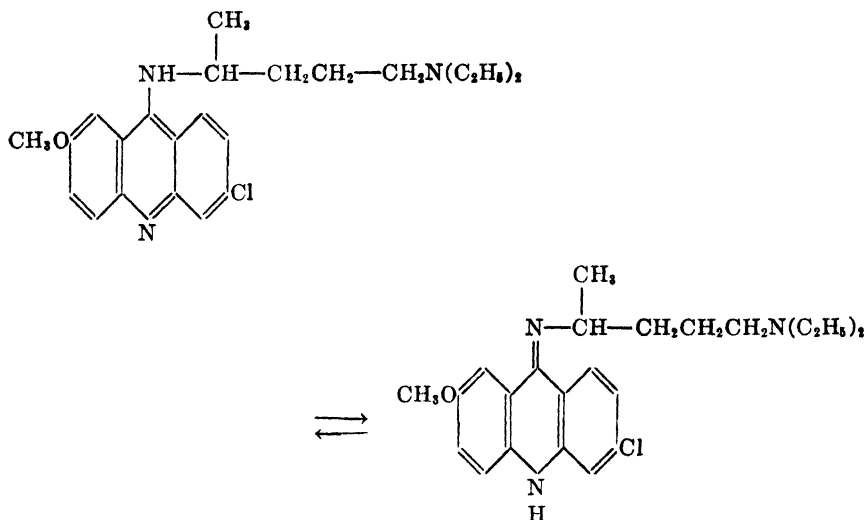


FIG. 1. The absorption of atabrine dihydrochloride at pH 2.10 (Curve 1) and pH 8.55 (Curve 2). $\epsilon \times 10^{-3}$ is the millimolecular extinction coefficient.

Fig. 1) with a band at 3430 Å. and a broad band in the visible region with maxima at 4250 and 4450 Å. As the pH of the solution was increased, the absorption began to change. The change was pronounced at 7.6 and complete at pH 8.55. At the latter pH there were small bands at 3420 and 3600 Å. and a band with a single maximum at 4200 Å., as shown in Fig. 1. The absorption curves obtained at all pH levels passed through isobestic points at 3530 and 4080 Å.; atabrine is therefore tautomeric and the tautomerism, which involves the chromophoric acridine ring, is limited to two molecular species.² Since 2-methoxy-6,9-dichloroacridine does not exhibit a similar pattern, the changes produced by raising the pH of the atabrine solution suggest the following tautomerism.

² Prideaux, E. B. R., *J. Soc. Chem. Ind.*, **45**, 678 (1926). Clark, W. M., *The determination of hydrogen ions*, Baltimore, 3rd edition, 154 (1928).



After the elution of atabrine from the column mentioned above, the strongly adsorbed band of brown material at the top of the column was removed mechanically, and discarded. The orange-yellow band was eluted with acidulated water. The colored material in this acid eluate is weakly basic. It may be extracted from basic solutions with benzene, and may be extracted from benzene with acids. Solutions of the material do not fluoresce appreciably in ultraviolet light, and while the solutions are yellow in color, absorption data suggest that the materials in this eluate are not acridines. We have not investigated this material beyond this point, but the acid eluate appears to be of interest because it does not occur in normal dog urine. The materials in this eluate may be produced by an aberrant pigment metabolism, or they may be atabrine detoxication products in which the acridine ring was ruptured.

Fraction B₁—The alkaline solution containing Fraction B₁ was washed with benzene; the pH was adjusted to 8.4 and the product was extracted with benzene. The benzene solution, dried briefly over anhydrous sodium sulfate, was allowed to percolate through a column of alumina 20 cm. in length and 8 mm. in diameter. Fraction B₁ was retained as an orange band at the top of the column. The chromatogram was developed with benzene containing 50 volumes per cent of isoamyl alcohol, and Fraction B₁ passed into the receiver. An equal volume of benzene was added to the eluate and the basic acridine was extracted from the organic phase with 0.5 N hydrochloric acid.

When exposed to ultraviolet light, acidic solutions of Fraction B₁ emitted a green fluorescence similar to that observed with solutions of atabrine, but,

unlike the latter, when the solution was made alkaline the color changed from yellow to orange and the solution then gave a distinctive yellow fluorescence in ultraviolet light.

Purified further by repetition of the distribution and chromatographic techniques, aqueous solutions of the product were examined spectrophotometrically. The concentration of the product in 0.5 N hydrochloric acid was determined by the colorimetric intensity measured at 4250 Å. and the data (shown in Fig. 2) are expressed in terms of atabrine dihydrochloride. The data obtained (Curve 1) are similar to those obtained with comparable

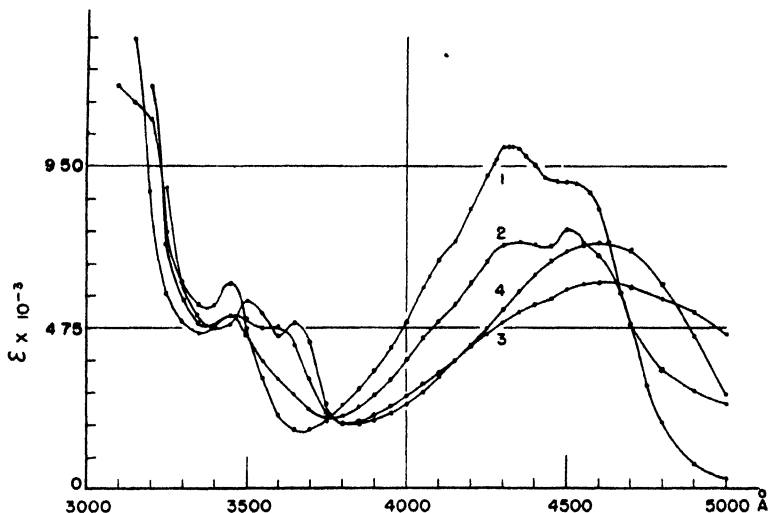


Fig. 2. The absorption of Fraction B₁ in 0.5 N hydrochloric acid (Curve 1), at pH 7.51 (Curve 2), at pH 8.58 (Curve 3), and in 2.5 N sodium hydroxide (Curve 4). ϵ is computed, arbitrarily, on the assumption that equimolar solutions display the same extinction coefficient at 4250 Å. as atabrine dihydrochloride. The data for Figs. 3 and 4 were obtained by similar treatment.

solutions of atabrine, indicating the acridine nature of Fraction B₁. Acidic solutions of Fraction B₁ exhibited maxima at 3450 and 4330 Å. with a suggestion of a maximum at 4500 Å. Different samples of Fraction B₁ always gave identical data over the range of 3300 to 5000 Å.

Maintaining the Fraction B₁ concentration constant, but increasing the pH to 7.02, produced no appreciable changes in the absorption, but when the pH was increased to 7.51 (Curve 2) the absorption was altered, and at pH 8.58 (Curve 3) there was a pronounced shift to the longer wave-lengths in the visible portion of the spectrum. The change appeared to be complete when the product was examined in 2.5 N sodium hydroxide (Curve 4). In this solution maxima were observed at 3500, 3650, and 4600 Å. The first

two bands correspond to those observed with alkaline solutions of atabrine, but the band in the visible region was shifted to longer instead of shorter wave-lengths. While it would seem that there is an invariant point of 3750 Å., there are no isobestic points around 3500 and 4650 Å. in Fig. 2. Thus the changes in the absorption, produced by increasing the pH of the solution, require more than one type of intramolecular rearrangement involving the acridine ring.

Certain conclusions regarding the nature of Fraction B₁ may be drawn from the absorption and distribution data. Fraction B₁ is about as basic as atabrine, indicating that the basic side chain linked to the 9-carbon atom is relatively unchanged. At least the amino grouping attached to the ring is intact. Thus, the product is capable of the same tautomerism exhibited by atabrine. Unlike atabrine, however, Fraction B₁ forms a sodium salt. Since the changes in the absorption are not due to decomposition and are complete only in strongly alkaline solution, the anionic grouping in Fraction B₁ is a weak one, and is presumably phenolic. The position of the phenolic group is unknown. It may appear in the molecule as the result of hydrolysis, *in vivo*, of the 2-methoxy group.

Fraction B₂—The isoamyl alcohol eluate containing Fraction B₂ was further purified as follows: 4 volumes of benzene were added to the alcohol and the basic acridine was extracted with 0.5 N hydrochloric acid. The acidic phase was washed twice with benzene to remove traces of isoamyl alcohol. The pH of the aqueous phase was increased to 8.4 and then to above 9.6 and the solution was washed with benzene after each pH increment to remove possible traces of atabrine and Fraction B₁. The product was then extracted with small quantities of isoamyl alcohol. Benzene was added to the combined isoamyl alcohol extracts to give a benzene solution containing 10 volumes per cent of isoamyl alcohol. After being briefly dried with anhydrous sodium sulfate, the solution was allowed to percolate through a column of alumina 40 cm. long and 12 mm. in diameter. The chromatogram was developed with the same solvent and the product was eluted with isoamyl alcohol. The eluate was diluted with 4 volumes of benzene and the product was extracted from the aqueous phase with 0.5 N hydrochloric acid.

After further purification by repetition of the distribution and chromatographic techniques, solutions of Fraction B₂ were examined spectrophotometrically. In the data for Curve 1 (Fig. 3) the concentrations are expressed in terms of atabrine. In 0.5 N hydrochloric acid Fraction B₂ gave a strong band at 3530 Å. and a broad band in the visible region with maxima at 4080 and 4300 Å. The character of the curve is similar to that obtained with solutions of atabrine, indicating the acridine nature of Fraction B₂. The absorption of the solution was not altered by increasing

the pH to 2.98. The change was significant at pH 4.95 (Curve 2), pronounced at pH 6.15 (Curve 3), and complete at pH 7.57 (Curve 4). Thus, Fraction B₂ is a weaker base than atabrine. Further, the changes in the absorption produced with increasing pH are quite different from those observed in comparable experiments with atabrine. In alkaline solution Fraction B₂ exhibits no detail in the near ultraviolet and a single strong band at 3970 Å. All four curves shown in Fig. 3 pass through common

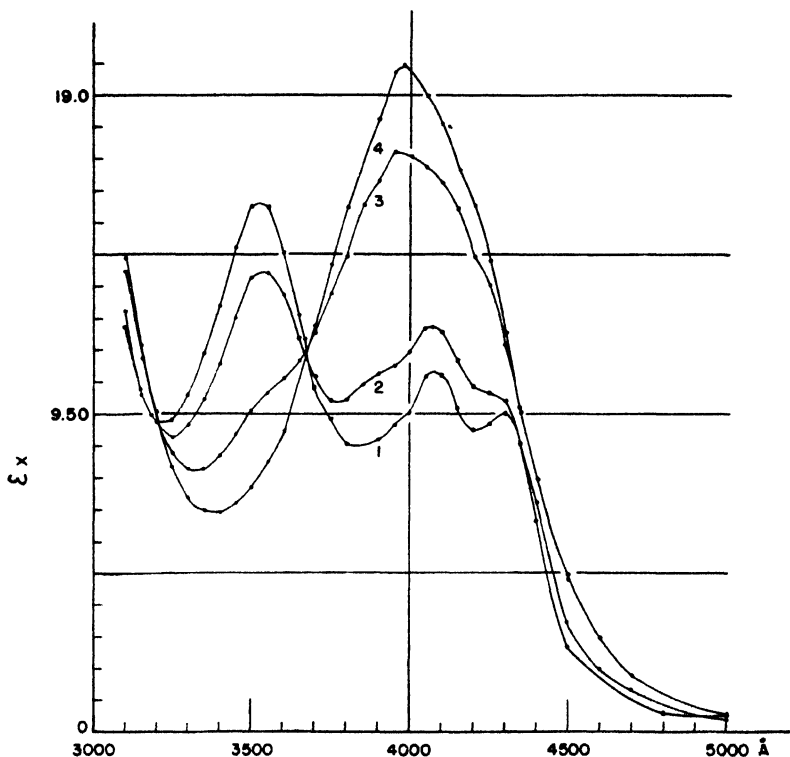


FIG. 3. The absorption of Fraction B₂ in 0.5 N hydrochloric acid (Curve 1), at pH 4.95 (Curve 2), at pH 6.15 (Curve 3), and at pH 7.57 (Curve 4).

points at 3210 and 3680 Å.; hence there is only one substance contributing to the absorption. This substance is capable of existing in two molecular forms depending upon the pH, and this tautomerism is presumably dependent upon the presence of the substituted 9-amino group. These observations suggest that, among other things, a partial degradation of the basic side chain may occur in the course of the conversion of atabrine to Fraction B₂ in the dog.

Fraction C—After the elution of Fraction C from the chromatogram, the

eluate was acidified and washed with benzene. The pH of the eluate was increased to 8.4 and then to a value above 9.6 and the aqueous phase was extracted with benzene after each pH increment. The product was extracted with comparatively large volumes of isoamyl alcohol. 4 volumes of benzene were added to the extract and the acridine was extracted from the organic phase with 0.5 N hydrochloric acid.

After repetition of the distribution and chromatographic procedures, solutions of Fraction C in 0.5 N hydrochloric acid were examined spectrophotometrically (Curve 1 of Fig. 4). Identical absorption data were found with all samples of Fraction C obtained, and these data are sufficiently similar to those obtained with atabrine to indicate the acridine nature of Fraction C. There is a band at 3450 Å. and a broad band in the visible

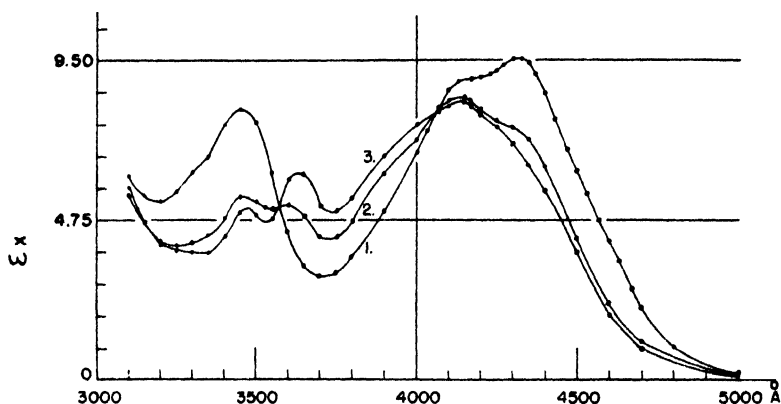


Fig. 4. The absorption of Fraction C in 0.5 N hydrochloric acid (Curve 1), at pH 8.60 (Curve 2), and at pH 9.57 (Curve 3).

region with a suggestion of a maximum at 4150 Å. and a distinct maximum at 4320 Å. The absorption was not changed appreciably when the pH was increased to 6.0. At pH 8.60 there were significant changes (Curve 2) which were complete at pH 9.57 (Curve 3). Thus, Fraction C is a stronger base than atabrine. The curves shown in Fig. 4 pass through common points at 3570 and 4080 Å., indicating the presence of only two molecular species in the solutions examined. Since the absorption of Fraction C is qualitatively similar to the absorption of atabrine in both acidic and alkaline solutions (compare Figs. 1 and 4), the molecular structures requisite to these absorption patterns are essentially unaltered in the conversion of atabrine to Fraction C in the dog. Apparently one or more polar groupings are present in Fraction C, since its free base is comparatively water-soluble, but this group (or groups), presumably phenolic in nature, does not influence the tautomerism appreciably.

Accumulated samples of Fraction C in 0.5 N hydrochloric acid were concentrated *in vacuo* at temperatures below 30°. The product was extracted with isoamyl alcohol after the concentrate was made strongly alkaline. The alcoholic extract was dried and saturated with hydrogen chloride and the product was precipitated by the addition of ether. After several recrystallizations from anhydrous isoamyl alcohol the product appeared as a fine yellow powder. The product contained 1.8 per cent ash and resisted purification. It darkened at 154° and melted with decomposition at 162–165°. 5 mg. of this product in 1 cc. of water were treated with 1 cc. of a saturated solution of picric acid. The picrate precipitated on standing. On recrystallization from alcohol, the picrate appeared as a crystalline powder which sintered at 152° and sublimed at 160–165°. Attempts to identify Fraction C were discontinued at this point.

Dr. Albert Seeler of the Merck Institute kindly examined purified concentrates of Fraction C and reported that these showed no schizonticidal activity when tested against a species of avian malaria at dose levels of 20 and 100 mg. per kilo of body weight. Further, the toxicity of these concentrates was greater than that of atabrine.

DISCUSSION

The present investigation was limited to the study of the isoamyl alcohol-soluble acridine bases excreted by way of the urinary system following oral administration of atabrine. It is clear that other solvents may extract additional products. Preliminary experiments involving extraction after hydrolysis have indicated that only small, if not negligible, amounts of the drug are excreted as water-soluble acridines of the ethereal sulfate or glucuronide type, but no exhaustive attempt was made to study the excretion of such products. Nor were any attempts made to seek products in which the acridine ring was ruptured, although by far the larger portion of the drug is presumably degraded to this extent because only about 5 per cent of the drug is recoverable in forms retaining the intact acridine ring.

In spite of these many restrictions imposed upon the scope of the investigation, the results reported here indicate that the urinary excretion of atabrine in the dog is quite complex. It has been shown that at least four acridine derivatives appear in the urine. One of these has been identified as unchanged atabrine. The remaining three products have been characterized spectrophotometrically; various properties have been noted and limited conclusions regarding functional groupings have been drawn. There is also a urinary fraction, not excreted normally by the dog, which does not appear to be composed of acridines. This fraction may include atabrine derivatives in which the acridine ring is no longer intact, or it may

appear in the urine as the result of an aberrant pigment metabolism induced by the drug.

The urinary excretion of atabrine varies in different animal species. The dog excretes about 5 per cent of the drug, and of the acridines eliminated, about 25 per cent appears as atabrine; both Fractions B₁ and B₂ appear to the extent of 2 to 5 per cent and the remainder is eliminated as Fraction C. Adult rats, given 25 mg. per kilo of body weight of the drug by stomach tube eliminated about 2 per cent of the drug over a 48 hour period. Of the acridines excreted, over 90 per cent was unchanged atabrine. No detectable amounts of Fraction B₁ were present and only 2 to 5 per cent of the acridines was eliminated as Fraction B₂ and C. A random sample of urine obtained from a human subject who received 700 mg. of the drug

TABLE I

Absorption of Various Acridines in 0.5 N Hydrochloric Acid

The values for the latter four substances were determined by the tinctorial intensity measured at 4250 Å. and the results are expressed in terms of atabrine. The symbol $\pm$ signifies that there was only a suggestion of a maximum at the given wave-length. λ is given in Å.

Substance	λ_1	$\epsilon \times 10^{-3}$	λ_2	$\epsilon \times 10^{-3}$	λ_3	$\epsilon \times 10^{-3}$	λ_4	$\epsilon \times 10^{-3}$
Atabrine.....	3420	4.87	4250	9.37	4450	8.94		
2 - Methoxy - 6 - chloro- 9-aminoacridine.....	3400	4.48	4110	6.03	4320	5.04		
2 - Methoxy - 6,9 - di- chloroacridine.....	3550	8.40	3700	16.6	4250	5.67	± 4450	5.19
Fraction B ₁	3450	6.00	4330	10.0	± 4500	8.99		
“ B ₂	3530	15.7	4080	10.7	4300	9.46		
“ C (dog).....	3450	7.99	± 4160	8.94	4330	9.55		
“ “ (rabbit).....	3400	4.54	4200	9.32	4450	8.61		

contained about as much Fraction C as unchanged atabrine. The concentrations of Fractions B₁ and B₂ were not determined. Pooled samples of urine obtained from rabbits 5 hours after the oral administration of 100 mg. of atabrine per kilo of body weight contained about 1 per cent of the dose administered. Of this, the output of Fraction C was approximately 5 times the output of atabrine, and there was no Fraction B₁ or B₂ in the sample.

The separations used in this work are based upon the solubility, distribution, and chromatographic behavior of the different excretion products. It is possible that different substances could satisfy these criteria, and that a given product obtained from one animal species may not be the same as the comparable product obtained from another species. This possibility was investigated spectrophotometrically. The substances characterized

as atabrine are the same in both species, but, as shown in Table I, the acridines excreted by the dog and rabbit and characterized as Fraction C are not identical.

SUMMARY

This study, limited to those substances extracted from alkaline solution with isoamyl alcohol, indicates that the urinary excretion of atabrine is quite complex. At least four acridine derivatives appear in the urine of the dog; of these, one has been identified as unchanged atabrine. The remaining three have been characterized spectrophotometrically. Various properties have been noted and limited conclusions regarding their functional groupings have been drawn. There is also a urinary fraction not normally excreted by the dog which does not appear to be composed of acridines. Comparison of the acridines eliminated by the rat, rabbit, dog, and man indicates that the urinary excretion of atabrine varies in different animal species.

AN ADDITION TO THE MARSHALL METHOD FOR THE DETERMINATION OF THE SULFONAMIDES IN BLOOD

By JOHN V. SCUDI AND VIOLA C. JELINEK

(From the Research Laboratories of Merck and Company, Inc., and the Merck Institute for Therapeutic Research, Rahway, New Jersey)

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The method of Bratton and Marshall¹ possesses an excellent sensitivity, but, since the length of the column of most photoelectric colorimeters is

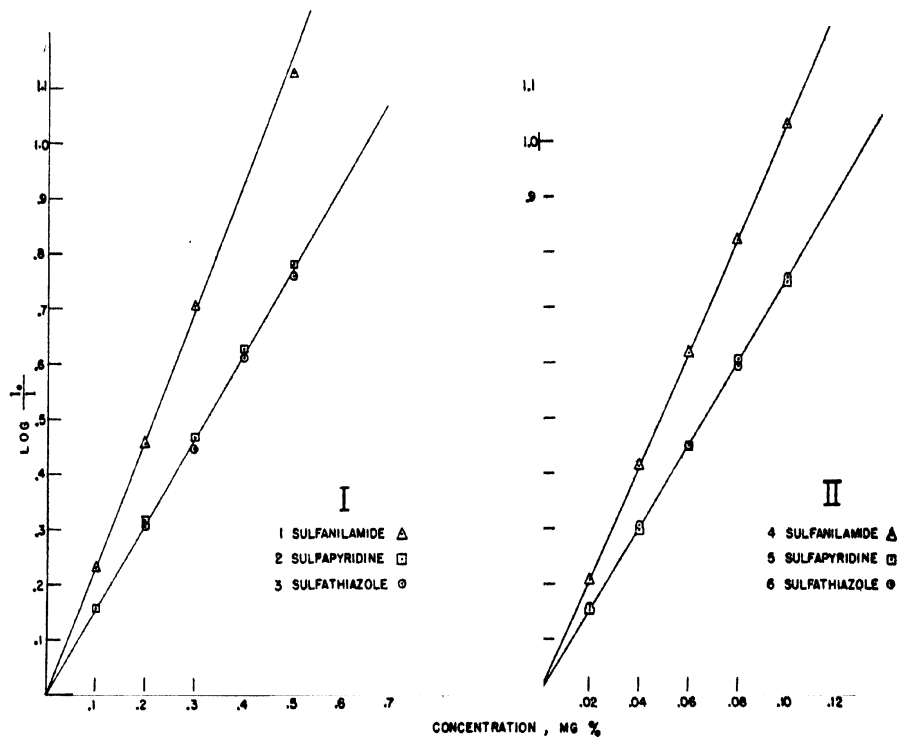


FIG. 1. (I) Varying concentrations of the sulfonamide drugs as measured by the Marshall method. (II) Lower concentrations measured in *n*-butanol following extraction.

fixed, and since low blood concentrations are frequently encountered, it is often impossible to measure the color developed in highly diluted blood filtrates. A simple means whereby the color can be concentrated is there-

¹ Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, **128**, 537 (1939).

fore desirable. Reduction of the blood dilution is not satisfactory since, as Bratton and Marshall¹ have shown, the sulfonamides and more particularly the heterocyclic sulfonamides are adsorbed by protein precipitates, and this adsorption varies inversely as the dilution. The desired concentration has been effected, without alteration of the Marshall method, by extracting the final azo color with small volumes of butanol.

Solutions of sulfanilamide, sulfathiazole, and sulfapyridine were analyzed¹ at concentrations ranging from 0.1 to 0.5 mg. per cent. The colors were measured in a 1 cm. cuvette at 5400 Å. in the Beckman spectrophotometer with the results shown in Fig. 1. The slopes of Lines 1, 2, and 3 are inversely proportional to the molecular weights of the drugs. The same experiments were repeated but only one-fifth of the respective drug concentrations was used. 5 minutes after the azo color was produced, the colored solution (13 cc.) was extracted with 3 cc. of *n*-butanol.² The colored butanol phase was drawn off, chilled in ice water, and centrifuged to prevent clouding by undissolved water. The colors, measured as usual,³ gave Lines 4, 5, and 6, in Fig. 1. The slopes indicate that only 90 per cent of the sulfanilamide dye was extracted but the extraction was complete in the case of the heterocyclic sulfonamides. These data indicate that the colors obey Beer's law, that the reproducibility of the method remains unaltered, and that the sensitivity of the method is increased by a factor of 5. With 20 cc. of filtrate this factor is increased to 10. This addition to the Marshall method has been satisfactorily used in routine blood analyses.

² Trichloroacetic acid as used in the Marshall method for blood should not be replaced by hydrochloric acid as in the method for urine, since this alters the distribution ratios.

³ We examined the absorption of aqueous and butanol solutions of these dyestuffs over the range 4500 to 6000 Å. Maxima were observed at 5450 Å. in all cases. Hence, the standard No. 540 filter may be used.

THE METABOLISM OF ACETALDEHYDE WITH ACETOIN FORMATION

By ELMER STOTZ,* W. W. WESTERFELD, AND ROBERT L. BERG

(From the Biochemical Laboratory of the McLean Hospital, Waverley, Massachusetts, and the Department of Biological Chemistry, Harvard Medical School, Boston)

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Acetaldehyde is a primary product of alcohol metabolism in the animal body. Together with alcohol, it comprises the equilibrium system activated by the alcohol dehydrogenase system (1), and its concentration in the blood, particularly in man, rises many fold after alcohol administration (2). For this reason it merits attention, but it is probably of further importance in the general scheme of carbohydrate metabolism. The intermediate formation of acetaldehyde in the decarboxylation of pyruvic acid by yeast was early established (3), and the study of a diphosphothiamine enzyme system from heart muscle by Green *et al.* (4) strongly suggests that pyruvic acid is first decarboxylated to acetaldehyde and is then further condensed with a second molecule of pyruvic acid to yield acetoin (acetylmethylcarbinol) according to the reactions



Green *et al.* found that in the presence of the enzyme the rate of acetoin formation from pyruvate was increased 4-fold if acetaldehyde was also present, that from a given amount of pyruvate the yield of acetoin in the presence of acetaldehyde was twice that obtained with pyruvate alone, and that when the acetaldehyde was replaced by propionaldehyde the corresponding acetylmethylcarbinol was formed. The production of acetaldehyde by tissues autolyzing in the presence of an aldehyde fixative (5–8) probably represents an interruption of the acetoin-forming reaction through fixation of the acetaldehyde first produced (9).

Special attention was directed to acetoin as a product of acetaldehyde metabolism by the work of Green *et al.* and by our previous report that pyruvate administration produces an acceleration of alcohol removal from the blood (10). Acetoin formation from pyruvate was earlier demonstrated in minced heart muscle by Gorr (9), and in skeletal muscle, liver, and kidney by Tanko *et al.* (11). The latter workers also noted that acetaldehyde increased the rate of acetoin formation from pyruvate. Our

* Present address, New York State Agricultural Experiment Station, Cornell University, Geneva, New York.

object has been to study the quantitative relationships in the acetaldehyde-pyruvate condensation system of brain tissue with the micromethods for acetaldehyde (2) and acetoin (12) developed in this laboratory. The application of these methods has made it possible to compare the influence of various substances on the same tissue sample under the controlled conditions afforded by use of the Warburg respirometer. This report includes a study of this reaction in thiamine-deficient brain and finally the demonstration of acetoin formation *in vivo* after the injection of acetaldehyde.

Experiments in Vitro

General Technique—Both rats and pigeons were used in these studies. In either case the brain was quickly removed from the decapitated animal, freed of major portions of white matter, weighed, and homogenized in 0.9 per cent saline. The suspension was further diluted so that each ml. contained 250 mg. of tissue. 1 ml. of this suspension was immediately pipetted into Warburg flasks already containing phosphate buffer and the substances to be tested. Acetaldehyde (100 to 300 γ in 0.3 ml.) was placed in the side arms, the total volume of fluid in the flasks was 3.3 ml., and the concentration of phosphate buffer was 0.05 M of pH 7.4. After adequate temperature (37°) or gas equilibration and closing of the taps, the acetaldehyde solution was admitted to the main chamber of the vessels. At this time, noted as zero, a representative flask was removed, and the contents carefully rinsed into a chilled graduated centrifuge tube. The mixture was deproteinized by the addition of 1 ml. of 10 per cent sodium tungstate and 1 ml. of $\frac{2}{3}$ N sulfuric acid, diluted to exactly 10 ml., and centrifuged. Changes in the concentration of substances under investigation were then calculated by the differences found between the zero time control flask and the other flasks similarly treated after 1 hour of incubation. 1 ml. of the filtrate suffices for the determination of acetaldehyde according to a previously described colorimetric method (2) and the remaining filtrate for the determination of acetoin (12). In those experiments in which pyruvate was determined, the contents of the flask were deproteinized with trichloroacetic acid (2 per cent final concentration) and the method of Bueding and Wortis (13) utilized. Acetaldehyde does not interfere in the pyruvate procedure.

Acetaldehyde and Pyruvate Disappearance—Because of the volatility of acetaldehyde, preliminary experiments were carried out to determine the extent of the losses due to this factor. When the customary 90 to 120 γ of acetaldehyde in 3.3 ml. of saline buffer were used, a volatilization loss of 5 to 8 γ occurred during the 1 hour period. The relatively small volatilization losses were determined frequently and subtracted from the values for acetaldehyde that disappeared in the presence of tissue.

The disappearance of acetaldehyde and pyruvate individually or when both were incubated with the homogenate of rat brain was determined. The results of such experiments are recorded in Table I and are strictly comparable, since all were determined simultaneously and on identical aliquots of a homogenate prepared by pooling three rat brains. The data show that (1) brain tissue metabolizes acetaldehyde under these conditions, (2) the amount which disappears, with or without the addition of pyruvate, is increased by a higher initial acetaldehyde concentration, (3) the addition of pyruvate greatly accelerates acetaldehyde disappearance, and conversely (4) addition of acetaldehyde accelerates pyruvate disappearance.

TABLE I

Acetaldehyde and Pyruvate Disappearance in Homogenates of Rat Brain

250 mg. of rat brain, total volume 3.3 ml.; saline-phosphate buffer 0.05 M, pH 7.4; incubation 1 hour at 37°.

Sample No.	Acetaldehyde added	Pyruvate added*	Acetaldehyde disappeared	Pyruvate disappeared*
	γ	γ	γ	γ
1	110	0	20	
2	110	300	48	
3	110	600	62	
4	110	900	66	
5	320	0	52	
6	272	600	95	
7	540	600	181	
8	0	600		414
9	110	600		502
10	272	600		512

* Calculated as pyruvic acid.

pearance. The latter effect is less striking, however, owing to the rapid pyruvate metabolism present without added acetaldehyde.

The simultaneous disappearance of pyruvate and acetaldehyde was not due to an oxidation-reduction reaction between the two, since (a) alcohol formation could not be detected, and (b) there was no greater lactate formation in the presence of both pyruvate and acetaldehyde than with pyruvate alone. Alcohol was determined in the distillate of a tungstic acid filtrate by a dichromate reduction method (10) after correction for the acetaldehyde present. Lactate was measured by the method of Barker and Summerson (14) after removal of acetaldehyde by distillation.

Free acetaldehyde could not be detected during active pyruvate disappearance when the latter was the only substrate added to the tissue.

Acetoin Formation.—Analyses for acetoin were performed after the usual

1 hour incubation of rat brain homogenate with acetaldehyde under various conditions. These conditions included (1) the use of a nitrogen atmosphere during incubation, (2) the addition of diphosphothiamine, and (3) the use of glucose and lactate as precursors of pyruvate. The more essential points investigated were again combined into a single experiment on a homogenate prepared by pooling three brains, so that the results would be strictly comparable. These are recorded in Table II.

The highest yields of acetoin and correspondingly the greatest disappearance of acetaldehyde occurred in the presence of pyruvate and

TABLE II
Formation of Acetoin in Homogenates of Rat Brain

250 mg. of rat brain, total volume 3.3 ml.; saline-phosphate buffer 0.05 M, pH 7.4; incubation 1 hour at 37°.

Sample No.	Acetalde- hyde added	Other substances added*		Diphospho- thiamine added	Acetalde- hyde disappeared	Acetoin formed
	γ		mg.	γ	γ	γ
1	104			0	16	0
2	104			20	22	9
3	104	Pyruvate	0.6	0	51	68
4	104	"	0.6	20	71	94
5†	104	"	0.6	20	76	100
6	104	Lactate	6.0	20	51	70
7†	104	"	6.0	20	30	38
8	104	Glucose	6.0	20	43	59
9†	104	"	6.0	20	32	41
10		Pyruvate	0.6	20		0
11		"	6.0	20		11
12		Glucose	6.0	20		0
13		Lactate	6.0	20		0

* Pyruvate and lactate are calculated as the free acid.

† Nitrogen-filled flasks.

diphosphothiamine. The substantial effect produced by added diphosphothiamine demonstrates the rôle played by this coenzyme in the acetaldehyde-pyruvate condensation, and proves that the amount of the coenzyme available in the homogenate of normal rat brain is not sufficient for maximum activity of the enzyme. Whether or not the insufficiency of coenzyme for this reaction exists in the intact tissue cannot be directly inferred, since homogenization may produce a "dilution effect" or allow for destruction of coenzyme. Acetoin formation from acetaldehyde without added pyruvate is very small and can only be detected regularly with the stimulation provided by added diphosphothiamine. It must be pointed out that the yield of acetoin in this case is at the lower limits of detection by the method employed and hence represents only qualitative evidence

for its presence rather than quantitative estimation. It is likely, in view of the other results, that this formation of acetoin represents largely a reaction of the acetaldehyde with pyruvate from residual precursors such as glucose and lactate, although the enzyme from heart muscle (4) is also able to cause a slow condensation of 2 molecules of acetaldehyde to form acetoin. A similar small yield of acetoin was observed when pyruvate was added without acetaldehyde, but only if a high concentration was employed.

The yield of acetoin from the combination of acetaldehyde and pyruvate, whether under aerobic or anaerobic conditions, indicates a nearly complete stability of the acetoin formed. The acetoin figures in Tables II to IV remain uncorrected for the approximately 15 per cent loss of acetoin inherent in the chemical determination (12). With this factor taken into account, some 80 per cent of the acetaldehyde which disappears according to the reaction $\text{CH}_3\text{CHO} + \text{CH}_3\text{COCOOH} = \text{CH}_3\text{—CO—CHOH—CH}_3 + \text{CO}_2$ is recovered as acetoin. *dl*-Acetoin was entirely stable in this type of brain preparation.

Glucose and lactate were tested in the system to determine whether they could effectively supply pyruvate for acetoin synthesis from acetaldehyde. These results are likewise recorded in Table II. Neither glucose nor lactate, even at concentrations higher than that of pyruvate, was as efficient as the latter but nevertheless led to considerable acetoin synthesis. The formation of acetoin from glucose and acetaldehyde was somewhat greater aerobically than anaerobically. In the case of lactate, a much greater acetoin synthesis was observed aerobically.

Tests for diacetyl, even under conditions of maximum acetoin formation, were consistently negative.

The formation of acetoin from acetaldehyde and pyruvate offers a useful tool to determine which substances may give rise to pyruvate during their metabolism. Application of this has thus far shown that glucose and lactate are metabolized through pyruvic acid in brain.

Acetoin Formation in Brain of Normal and Thiamine-Deficient Pigeons—The marked sensitivity of the acetoin-forming enzyme system to a lack of thiamine was demonstrated in the brain tissue of normal and thiamine-deficient pigeons. Several birds were placed on a thiamine-deficient diet and tube-fed in a manner previously described by Swank and Bessey (15). Others were maintained on the usual grain diet. When a bird reached the stage of opisthotonus (13 to 16 days), it was sacrificed and the brain tissue tested simultaneously with that of a normal bird. The effect of added pyruvate and diphosphothiamine was tested on acetaldehyde disappearance and acetoin formation in the brains of five normal and five deficient pigeons. The results are summarized in Table III.

The findings within each group of pigeons are quite uniform, but the

differences between the groups are marked. The averages in each group may be compared for discussion. In considering acetaldehyde disappearance, there is noted (1) no essential difference in the two groups when acetaldehyde alone is added, (2) a 160 per cent increase in the normal brains when pyruvate is added and only a 75 per cent increase in the brains of thiamine-deficient pigeons, and (3) a 17.5 per cent further acceleration

TABLE III

Acetaldehyde Disappearance and Acetoin Formation in Brain of Normal and Thiamine-Deficient Pigeons

250 mg. of pigeon brain, total volume 3.3 ml.; saline-phosphate buffer 0.05 M, pH 7.4; incubation 1 hour at 37°.

	Pigeon No.	Acetaldehyde disappearance*			Acetoin formation	
		Brain alone	With 0.6 mg. pyruvate†	With 0.6 mg. pyruvate + 20 γ diphosphothiamine	With 0.6 mg. pyruvate†	With 0.6 mg. pyruvate + 20 γ diphosphothiamine
Normal	1	30	74	80	44	52
	2	16	59	76	39	56
	3	24	65	70	40	52
	4	28	68	81	53	57
	5	30	75	90	53	57
Average.....		26	68	79	46	55
Thiamine-deficient	1	16	40	68	10	44
	2	22	24	60	0	54
	3	23	38	69	8	45
	4	25	45	73	11	50
	5	30	50	80	11	44
Average.....		23	40	70	8	47

* 104 to 110 γ were initially present.

† Calculated as pyruvic acid.

when diphosphothiamine is added to the normal brain as compared to a 75 per cent further increase in the brains of thiamine-deficient birds. A corresponding effect was noted on acetoin formation. In this case the normal brains showed an average formation of 46 γ of acetoin per 250 mg. of brain per hour as compared to 8 γ for the thiamine-deficient birds. Additional diphosphothiamine produced an increase in acetoin formation of only 20 per cent in the normal brains, while nearly a 6-fold increase was found in the thiamine-deficient tissue. The point seems proved from these

data that the acetaldehyde-pyruvate condensation with acetoin formation in brain tissue is dependent on diphosphothiamine and that tissue deficient in this factor is greatly stimulated with respect to this reaction by addition of the coenzyme.

The marked decrease in ability of the deficient tissue to metabolize acetaldehyde in the presence of pyruvate, in conjunction with the fact that even normal homogenate is not operating maximally with the usual complement of the vitamin, suggests the reaction as a possible basis for a sensitive functional test for thiamine deficiency. The measurement of very small quantities of acetaldehyde would facilitate the use of small amounts of tissue.

Experiments in Vivo

Several rats were injected with acetaldehyde (in normal saline) intraperitoneally in order to establish the desired conditions for demonstration of acetoin formation *in vivo*. A dose of 50 mg. per 100 gm. of body weight is practically certain to kill a rat within 10 minutes. The animal is usually limp in less than 3 minutes and later develops respiratory distress with gasping and finally death. On smaller doses an animal first develops an abnormal gait, and may walk in circles with the legs very limp and extended to the side. Equilibrium is readily lost and finally motion is difficult or impossible. The symptoms of acetaldehyde poisoning are very similar to those noted during alcoholic intoxication in rats. Most striking is the fact that it may take only a few minutes after the acute stage for the animal to become essentially normal again. This is consonant with the fact that during the acute stage the blood acetaldehyde may reach as high as 30 mg. per cent and 10 to 15 minutes later may be less than 1 mg. per cent. Whereas a single 100 mg. dose readily kills a 200 gm. rat, a similar animal may receive a 20 mg. dose at 15 minute intervals until at least a total of 250 mg. has been given without the animal showing any obvious effects at any time. 10 minutes after the last dose such a rat showed a blood acetaldehyde concentration of only 0.7 mg. per cent. It is apparent that acetaldehyde is quite toxic at higher levels but is rapidly metabolized.

It was not regularly possible to demonstrate acetoin formation in rats after a single dose of acetaldehyde. This could be demonstrated, however, if the rat received three successive large doses of acetaldehyde (20 to 30 mg. per 100 gm.) intraperitoneally and was sacrificed for blood collection 5 to 10 minutes after the last dose. Blood was collected from the severed neck of the animal and a 1:5 tungstic acid filtrate prepared. Acetaldehyde and acetoin were determined in aliquots of this filtrate. A summary of the results is recorded in Table IV.

Blood acetoin values as high as 2.7 mg. per cent may be reached with the

technique employed. If the animals were sacrificed 15 to 25 minutes rather than 5 to 10 minutes after the last acetaldehyde injection, the blood acetaldehyde values dropped to less than 1 mg. per cent and acetoin could no longer be detected. It is, therefore, indicated that the natural form of acetoin formed *in vivo* is rapidly metabolized. Acetoin formation could not be detected after small doses of acetaldehyde or after alcohol administration, the latter producing only low blood acetaldehyde values. Simultaneous administration of pyruvate with alcohol did not increase

TABLE IV
Formation of Acetoin in Vivo

The rats received three intraperitoneal injections at 10 minute intervals of the substance listed and were sacrificed 5 to 10 minutes after the last dose.

Rat weight	Substance injected	Blood acetaldehyde	Blood acetoin
gm.		mg. per cent	mg. per cent
108-283	None (controls)	0	0*
146-194	100 mg. Na pyruvate	0	0
203-242	50 " alcohol	0.18, 0.21, 0.16	0
206-270	50 " " + 100 mg. Na pyruvate	0.20, 0.28, 0.42	0
193	40 mg. acetaldehyde	3.6	0
118	40 " "	11.9	1.83
222	68 " "	14.7	2.00
219	66 " "	11.6	2.13
194	58 " "	9.0	1.97
210	52 " "	2.8	1.30
226	56 " "	1.1	0
143	40 " " + 90 mg. Na pyruvate	4.3	1.10
147	40 mg. acetaldehyde + 90 mg. Na pyruvate	9.0	2.74

* Blood acetoin values of 0 are actually <0.3 mg. per cent.

blood acetoin to detectable levels, nor when administered with acetaldehyde did it produce any obvious increase in acetoin formation or acetaldehyde disappearance.

DISCUSSION

The nearly quantitative formation of acetoin from acetaldehyde in the presence of pyruvate by brain homogenates indicates nearly complete stability of the acetoin in such a preparation. This has been a technical advantage in the demonstration of acetoin formation but of questionable value in leading to further understanding of its metabolism *in vivo*. It

seems likely that the failure of acetoin to be metabolized further at an appreciable rate depends on a dilution or destruction of a necessary component of the metabolic system involved.

Greenberg (16) injected *dl*-acetoin in dogs and found that the rate of its disappearance from the blood was a rather slow process. We have noted (unpublished experiments) a similar slow disappearance from the blood of rats, again after the injection of *dl*-acetoin. The *in vivo* experiments reported in this paper would indicate that the isomer synthesized from acetaldehyde *in vivo* is actually very rapidly metabolized. It could never be detected if more than a few minutes elapsed between the last injection of acetaldehyde and death of the animal. It seems probable, therefore, that it is the unnatural form of acetoin which is so slowly metabolized *in vivo*. Greenberg's conclusion concerning the slow rate of acetoin metabolism led him to the belief that alcohol does not pass through the acetoin stage in its metabolism, since acetoin did not accumulate during alcohol metabolism. The great increase of acetaldehyde in the blood after alcohol administration and the occurrence of alcohol dehydrogenase in the liver leave little doubt that acetaldehyde is a product of alcohol metabolism; and since we have shown that acetoin is formed after acetaldehyde administration, it seems likely that acetoin actually can arise from alcohol. Failure to detect it seems rather to depend on the rapid removal of the naturally formed isomer.

Bernhard (17) demonstrated the presence of deuterium in the acetyl group of acetylsulfanilamide excreted by rabbits receiving deuterioethyl alcohol and sulfanilamide. Doisy and Westerfeld (18) have more recently shown that acetoin is a precursor of the acetylating agent. The isotope studies of Fishman and Cohn (19) indicate, however, that neither pyruvate nor acetate can be the *direct* acetylating agent. The conclusions reached in this paper concerning the path of alcohol metabolism would, therefore, suggest the utilization of the acetoin pathway in the formation of the acetylating agent.

SUMMARY

1. Acetaldehyde is metabolized when incubated with rat brain homogenates. Its rate of disappearance is greatly accelerated by addition of pyruvate and further increased by addition of diphosphothiamine.
2. Pyruvate disappearance is also accelerated by the addition of acetaldehyde but the effect is not as pronounced owing to an initially high rate of pyruvate metabolism.
3. Acetoin (acetylmethylcarbinol) is formed nearly quantitatively in rat brain homogenates from acetaldehyde in the presence of pyruvate. The same factors which accelerate acetaldehyde disappearance also increase acetoin formation.

4. Brain tissue from thiamine-deficient pigeons shows a markedly lower ability to form acetoin from acetaldehyde and pyruvate and is stimulated in this respect to a greater extent than normal pigeon brain by diphosphothiamine.

5. Certain toxic manifestations of acetaldehyde in rats have been described and a procedure developed whereby acetoin formation from acetaldehyde was demonstrated *in vivo* by the appearance of acetoin in the blood.

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ALCOHOL METABOLISM IN THIAMINE DEFICIENCY

By ROBERT L. BERG, ELMER STOTZ,* AND W. W. WESTERFELD

(From the Department of Biological Chemistry, Harvard Medical School, Boston, and the Biochemical Laboratory of the McLean Hospital, Waverley, Massachusetts)

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A relationship of thiamine to alcohol metabolism is implied in the theory of intermediary acetoin formation (1), since the latter would be formed from pyruvate and acetaldehyde (arising from alcohol oxidation) by a diphosphothiamine enzyme system (2). Studies *in vitro* (3) showed that the metabolism of acetaldehyde and the formation of acetoin in brain brei were markedly decreased in thiamine deficiency; both were restored by the addition of diphosphothiamine. A need for thiamine in the metabolism of alcohol was clearly indicated by the clinical studies of Jolliffe *et al.* (4, 5) in which they found that chronic alcoholics showing symptoms of polyneuritis received enough dietary thiamine to metabolize the food eaten but an inadequate amount to metabolize both the food and the alcohol. No evidence for this relationship was found in rats, since alcohol administration actually delayed the onset of the polyneuritis syndrome (6).

From these considerations, it seemed possible to obtain direct evidence of a relationship between thiamine and alcohol metabolism by studying the latter during thiamine deficiency. However, the results have shown that the rate of alcohol metabolism is not decreased during thiamine deficiency, nor was any change noted in the effect of pyruvate in increasing the rate of alcohol metabolism (1). A previous study (7) showed that the oxidation of alcohol to acetaldehyde is normally the limiting reaction in alcohol metabolism; *i.e.*, the metabolic removal of acetaldehyde is much more rapid than its formation from the alcohol. The present study indicates that even during thiamine deficiency the rate of acetaldehyde metabolism is not sufficiently reduced to become the limiting factor in alcohol metabolism. This is not to be interpreted as necessarily indicating that thiamine is unessential for the metabolism of alcohol.

EXPERIMENTAL

Alcohol Metabolism in Normal Pigeons—Alcohol metabolism was studied in pigeons by measuring its rate of decrease in the blood over a period of 5 hours. The alcohol was administered by an intramuscular injection of 15 cc. per kilo of a 19 per cent (by volume) solution of alcohol. The blood

* Present address, New York State Agricultural Experiment Station, Cornell University, Geneva, New York.

alcohol concentration was determined (1) on 0.2 cc. portions of blood withdrawn by syringe from the wing veins at 1 hour intervals. Equilibrium was usually established in pigeons within 120 minutes of the alcohol administration, and the blood alcohol thereafter decreased as a linear function of time.

In Table I are summarized the alcohol oxidation rates obtained with a group of normal pigeons that had been fed a diet of mixed grain *ad libitum*, and a second group that had been force-fed a high carbohydrate diet for 10 days. The latter group was fed Diet III of Swank and Bessey (8),

TABLE I
Alcohol Metabolism in Normal Pigeons

Diet	Pigeon weight		Blood alcohol concentration at 0 time (by extrapolation)	Decrease of blood alcohol
	At start of diet	At time of determining alcohol curve		
	gm.	gm.	mg. per cent	mg. per cent per hr.
Mixed grain <i>ad libitum</i>		440	187	20
		430	178	14
		460	180	17
		484	190	17
		507	182	16
		348	179	17
Average				17
High carbohydrate diet, force-fed	508	529	179	24
	586	605	204	23
	496	480	184	18
	552	590	212	27
	430	453	185	31
Average				25

except that the yeast was not autoclaved and the diet was supplemented by the injection of 0.5 mg. of thiamine every 3rd day. The birds on a mixed grain diet oxidized alcohol at a rate that caused the blood alcohol to decrease an average of 17 mg. per cent per hour. This was increased to 25 mg. per cent per hour on the high carbohydrate diet. A high rate of alcohol oxidation was previously observed (9) in rats fed an all carbohydrate diet.

Alcohol Metabolism in Thiamine-Deficient Pigeons—Thiamine-deficient pigeons were obtained by the daily force feeding of the Swank and Bessey Diet III until opisthotonus appeared. An alcohol curve was run at the

time the birds were showing head retraction. They were then given daily injections of 0.5 mg. of thiamine intramuscularly for 7 days. The force feeding of the diet was continued as before, and at the end of the period of thiamine injections the rate of alcohol metabolism was again determined on each pigeon.

At the same time, an additional group of pigeons was fed the same diet and also injected with 0.5 mg. of thiamine daily from the beginning of the experiment to act as paired controls for some of the thiamine-deficient birds. After an interval corresponding to the time required to develop opisthotonus in the paired control, an alcohol curve was run. These pigeons were then continued on the same régime for an additional 7 days, and a second alcohol curve was run.

The data are summarized in Table II. From the average rates of 28.5 mg. per cent per hour during opisthotonus and 33 mg. per cent per hour after thiamine injection, it might appear that the rate was decreased in thiamine deficiency. However, a similar increase was obtained in the control group that had received adequate amounts of thiamine throughout. Moreover, the rate of 28.5 mg. per cent per hour during the deficiency period was as high as that observed in the control group. It is evident from these data that the rate of alcohol metabolism is more dependent upon the length of time the pigeons were fed a high carbohydrate diet than on the presence or absence of thiamine in that diet. Certainly thiamine deficiency did not decrease the rate of alcohol metabolism.

The absence of thiamine in the diet did not prevent the deposition of liver glycogen, even though the birds were losing weight. Values of 3 to 6 per cent liver glycogen were obtained by the usual method of analysis (10) in a series of pigeons with opisthotonus. Similar values were previously reported for normal and thiamine-deficient pigeons (11, 12) and show that in thiamine deficiency there are adequate carbohydrate reserves to maintain the rate of alcohol metabolism.

Alcohol Metabolism in Thiamine-Deficient Dogs—Three thiamine-deficient dogs were obtained by a preliminary feeding *ad libitum* of autoclaved dog chow for 5 weeks, followed by the feeding of 200 gm. of the Swank and Bessey Diet III daily by stomach tube for 5 to 19 days. The diet was supplemented by an injection of 25 mg. of calcium pantothenate each week. Marked thiamine deficiency was first indicated by vomiting of the food that had been fed by stomach tube, and this was followed within 1 or 2 days by partial paralysis of the legs, particularly the hind legs.

The effect of pyruvate administration on the blood alcohol curve was observed (a) before the dogs were placed on the diet, (b) while they were showing the paralytic symptoms of thiamine deficiency, and (c) once each week after thiamine had been administered. The dogs were relieved of

TABLE II
Alcohol Metabolism in Thiamine-Deficient Pigeons

	Dietary period preceding opisthotonus	Pigeon weight			Alcohol curve during opisthotonus		Alcohol curve after thiamine injection	
		At start of diet	At opisthotonus	After thiamine injections	Blood alcohol concentration at 0 time (by extrapolation)	Decrease of blood alcohol	Blood alcohol concentration at 0 time (by extrapolation)	Decrease of blood alcohol
	days	gm.	gm.	gm.	mg. per cent	mg. per cent per hr.	mg. per cent	mg. per cent per hr.
Thiamine-deficient pigeons	12	340	326	380	238	21	205	31
	12	451	437	477	267	33	236	35
	12	442	359	431	239	22	234	29
	14	496	396	446	228	23	219	38
	14	418	383	413	262	29	240	48
	15	440	428	507	270	27	274	38
	15	350	340	373	230	37	224	39
	18	451	384	460	259	27	222	24
	18	430	382	422	270	41	194	28
	19	377	310	380	225	26	196	27
	19	344	366	389	201	27	176	24
	23*	419	378	450	235	31	204	31
	25	397	340	382	193	26	226	38
	32†	375	352		221	27		
Average						28.5		33
	Dietary period preceding 1st alcohol curve	Pigeon weight			1st alcohol curve		2nd alcohol curve	
		At start of diet	At time of 1st alcohol curve	At time of 2nd alcohol curve	Blood alcohol concentration at 0 time (by extrapolation)	Decrease of blood alcohol	Blood alcohol concentration at 0 time (by extrapolation)	Decrease of blood alcohol
	days	gm.	gm.	gm.	mg. per cent	mg. per cent per hr.	mg. per cent	mg. per cent per hr.
Control pigeons	15	365	413	411	206	23	250	40
	18	427	452	478	214	22	209	29
	18	485	495	503	210	18	203	28
	19	445	489	474	267	32	207	27
	19	386	417	423	217	32	194	32
	20	461	430	468	223	25	262	30
	22	344	388	431	202	26	254	33
	23	472	554	560	265	34	272	36
Average						26.5		32

* Opisthotonus 3 days previously cured with 10 γ of thiamine.

† No opisthotonus developed; not included in the average of the group.

the deficiency symptoms, while the same force-fed diet was continued, by an initial injection of 20 mg. of thiamine followed by 10 mg. of thiamine every 2nd day thereafter for 2 weeks. The symptoms disappeared completely a few days after the first thiamine injection.

The effect of pyruvate administration on the blood alcohol curve was measured as previously described (1) except that the pyruvate was injected intravenously to avoid possible absorption complications during the deficiency. None of the thiamine-deficient dogs vomited the 20 per cent alcohol administered by stomach tube even though they had vomited their food the previous day. Blood pyruvate (13) and lactate (14) values were measured simultaneously with the determination of the rate of alcohol oxidation.

Results

Similar results were obtained on all three thiamine-deficient dogs. Fig. 1 shows the effect of pyruvate on the alcohol curve in one of these dogs during thiamine deficiency and after thiamine treatment for 1 week. Pyruvate was effective in increasing the rate of alcohol metabolism from 9 mg. per cent per hour to 19 mg. per cent per hour during thiamine deficiency, and from 15 mg. per cent per hour to 19 mg. per cent per hour after thiamine treatment. Thiamine deficiency did not prevent or impair the pyruvate effect in any one of the three dogs studied. Additional curves obtained during the succeeding weeks after restoration to the normal state did not show any significant differences.

The control rate of alcohol metabolism was higher after thiamine administration, but this is of doubtful significance because of the considerable variation that was encountered in the control rate of alcohol metabolism in the same dog from week to week. There was no obvious correlation of the rate of alcohol metabolism with previous treatment or dietary régime, except that dogs fasted 1 week had a slightly lower rate of alcohol metabolism than when they were well fed. In general, pyruvate administration increased the rates to 19 to 25 mg. per cent per hour irrespective of the initial control rate; pyruvate administered orally seemed to be somewhat more effective than when it was administered intravenously.

Blood Pyruvate and Lactate Changes—In the three thiamine-deficient dogs, the blood pyruvate was increased from a normal level of about 1.5 mg. per cent to 3.5 to 4.5 mg. per cent. The lactate-pyruvate ratio also changed from a normal value of about 8:1 to 5:1. After thiamine treatment for 1 week, the blood pyruvate fell to about 2 mg. per cent and the lactate-pyruvate ratio returned to 8:1. Further thiamine treatment produced little additional change either in pyruvate levels or the lactate-pyruvate ratio.

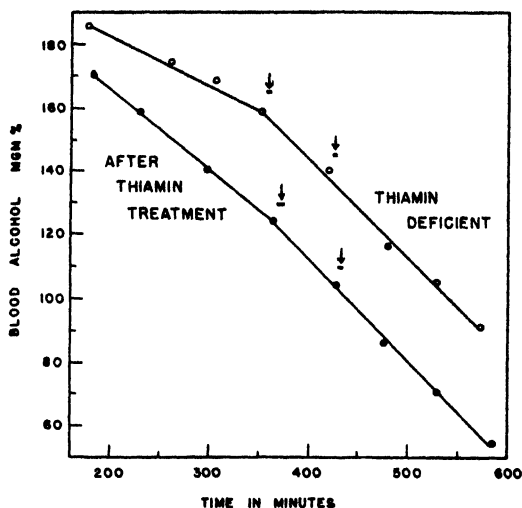


FIG. 1. The effect of intravenously injected pyruvate on alcohol metabolism during thiamine deficiency and after thiamine treatment. Dog 10M, weight originally 14.6 kilos, thiamine-deficient 9.0 kilos, after 1 week of thiamine treatment 9.3 kilos. 25 cc. of 95 per cent alcohol were given in a 20 per cent solution by stomach tube at zero time. 50 cc. of 10 per cent sodium pyruvate were injected intravenously at each arrow. Rates of decrease of blood alcohol in mg. per cent per hour, thiamine-deficient: control 9, after pyruvate 19; thiamine-treated: control 15, after pyruvate 19.

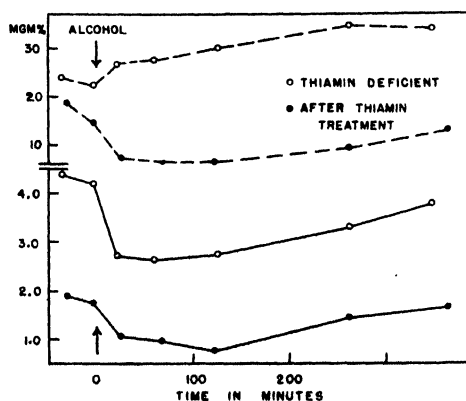


FIG. 2. The blood pyruvate and lactate response to alcohol feeding during thiamine deficiency and after thiamine treatment. Dog 10M, weight originally 14.6 kilos, thiamine-deficient 9.0 kilos, after 2 weeks of thiamine treatment 9.3 kilos. 25 cc. of 95 per cent alcohol were given in a 20 per cent solution by stomach tube at zero time.

Fig. 2 shows the changes in blood pyruvate and lactate after alcohol administration to a thiamine-deficient dog and to the same dog after 2 weeks of thiamine treatment. During the deficiency, the administration of alcohol caused a sharp fall in the blood pyruvate levels, but the blood lactate increased instead of showing a parallel fall. In all of the curves run after thiamine treatment, the alcohol administration caused a parallel fall in both the pyruvate and lactate.

DISCUSSION

The rate of alcohol metabolism was not decreased during thiamine deficiency. Normally alcohol metabolism is limited by the rate of its oxidation to acetaldehyde, and the metabolic removal of acetaldehyde is too rapid to affect this oxidation (7). A decrease in the rate of alcohol metabolism during thiamine deficiency could be expected only if the enzyme systems responsible for the metabolism of acetaldehyde (of which one is a diphosphothiamine enzyme) were depleted to such an extent that the removal of acetaldehyde became the limiting factor in alcohol oxidation. Obviously, this did not occur. Either the major part of the acetaldehyde is metabolized by enzymes not requiring diphosphothiamine for their action, or there is sufficient diphosphothiamine present even in severe thiamine deficiency to allow the metabolism of acetaldehyde to proceed at a faster rate than it can be formed from the alcohol.

Pyruvate was just as effective in increasing the rate of alcohol metabolism in thiamine-deficient dogs as it was in normals. This effect might be impaired in thiamine deficiency if it were due to a condensation of the pyruvate with acetaldehyde, as originally suggested (1). No change in the pyruvate effect on alcohol metabolism in thiamine deficiency would be anticipated if it were due to a coupled oxidation-reduction of the pyruvate and alcohol (7). The results are additional evidence in support of the latter explanation.

The blood pyruvate and lactate changes resulting from the thiamine deficiency in dogs were analogous to the results previously reported for pigeons (15). However, an explanation for the changes in these constituents observed after alcohol administration is not entirely clear. A decreased blood pyruvate would result from the dismutation reaction with alcohol (7), but the expected lactate rise was observed only during thiamine deficiency and not after restoration to normal. A parallel fall in blood pyruvate and lactate was also observed when alcohol was administered to dogs that were maintained on a normal diet. Some normal dogs showed very little change in either the blood pyruvate or lactate after alcohol ingestion, and this appeared to be correlated with low initial levels.

The symptoms of thiamine deficiency have occasionally been attributed

to the elevated blood pyruvate *per se*, and the delayed onset of polyneuropathy in thiamine-deficient rats fed alcohol (6) might, thereby, be attributed to a reduction in the blood pyruvate level by the alcohol. On the other hand, the intravenous injection of 100 cc. of 10 per cent sodium pyruvate into one of the thiamine-deficient dogs had no effect on the dog's ability to walk or on any of the other deficiency symptoms, even though the blood pyruvate rose from 5 mg. per cent to 139 mg. per cent after the injection and remained above 10 mg. per cent for at least 1 hour. It is more probable that the symptoms are caused by the inability of certain tissues (especially nervous tissue) to carry on a normal metabolism, which involves as an essential step the metabolism of pyruvate by a diphosphothiamine enzyme system.

SUMMARY

The concentration of blood alcohol decreased at an average rate of 17 mg. per cent per hour in pigeons fed a mixed grain diet *ad libitum* and 25 mg. per cent per hour when fed a high carbohydrate diet for 10 days. The rate of alcohol metabolism was not decreased during opisthotonus.

Pyruvate administered intravenously was equally effective in increasing the rate of alcohol metabolism during thiamine deficiency in dogs and after thiamine treatment.

Blood pyruvate was elevated in thiamine-deficient dogs, and the lactate-pyruvate ratio was decreased. The administration of alcohol to thiamine-deficient dogs decreased the blood pyruvate and increased the blood lactate; after thiamine treatment, the alcohol administration caused a parallel fall in both the blood pyruvate and lactate.

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STUDIES ON BILE ACID METABOLISM*

IV. SEPARATION AND IDENTIFICATION OF KETOCHOLANIC ACIDS FORMED DURING OXIDATION OF CHOLIC ACID BY ALCALIGENES FAECALIS

By WILLARD M. HOEHN, L. H. SCHMIDT, AND HETTIE B. HUGHES

(From the Research Laboratories, George A. Breon and Company, Kansas City,
Missouri, and the Institute for Medical Research, Christ Hospital, Cincinnati)

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A previous study (1) showed that catabolism of cholic acid took place in the cecum of the guinea pig through the action of a Gram-negative bacillus identified as *Alcaligenes faecalis*. Further experiments (2) showed that this organism, cultured aerobically in a simple synthetic medium containing cholic acid, oxidized this bile acid to ketocholanic acids. Quantitative studies (2) suggested a stepwise oxidation with successive formation of mono-, di-, and triketocholanic acids. The end-product, identified as 3,7,12-triketocholanic acid (dehydrocholic acid), was isolated from suitable digests in 85 per cent of theoretical yields. The intermediate reaction products (*i.e.*, the mono- and diketocholanic acids) were not isolated in pure form nor adequately identified. Crude acid mixtures containing these intermediates gave negative Zimmermann reactions, indicating the absence of a carbonyl group at C₃ (3). On the basis of this finding, it was tentatively suggested that the monoketo acid was either 3,7-dihydroxy-12-ketocholanic or 3,12-dihydroxy-7-ketocholanic acid, whereas the diketo acid was most likely 3-hydroxy-7,12-diketocholanic acid.

The intermediate reaction products have been more precisely identified in the present study. One of us (W. M. H.) has previously studied the separation and identification of ketocholanic acids formed during oxidation of cholic acid with chromic acid. The methods developed in this work (4) were applied to the present problem. Through use of these procedures, it has been shown that 3,12-dihydroxy-7-ketocholanic acid and 3-hydroxy-7,12-diketocholanic acid are two of the intermediate derivatives formed during bacterial oxidation of cholic acid to triketocholanic acid.

EXPERIMENTAL

A schematic outline of the methods used in the separation and identification is shown in the accompanying diagram. Details of the procedures are

* Reports of parts of this study were presented before the meeting of the American Society of Biological Chemists at Boston, April 1, 1942, and the American Chemical Society at Memphis, April 20, 1942.

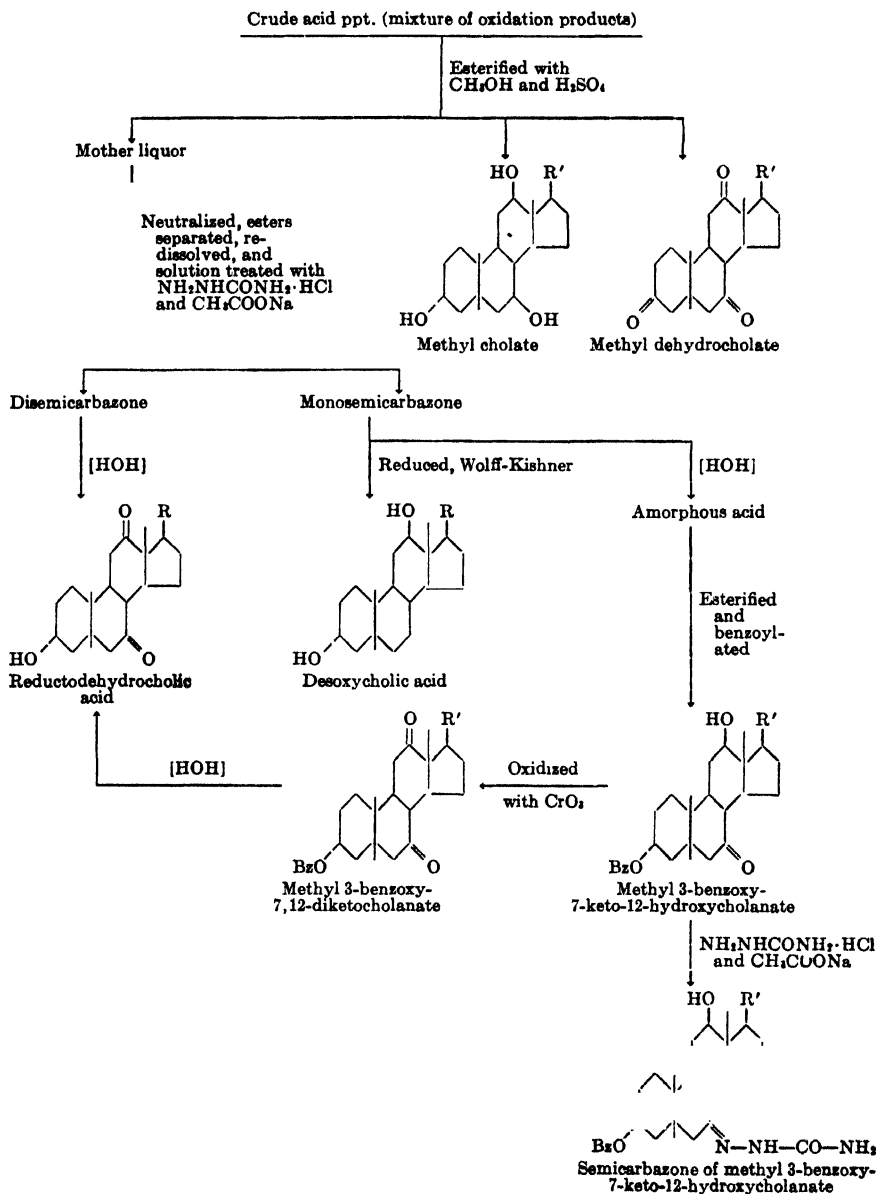
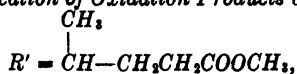
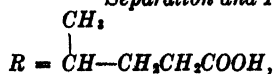
as follows: Crude acid precipitates, containing various amounts of unchanged cholic acid and intermediate oxidation products, were prepared from filtrates of cultures of *Alcaligenes faecalis* in media containing cholic acid. The methods of preparing the digests, as well as the procedures for determining cholic acid and keto acids, have been described heretofore (2, 5, 6).

Preparation of Methyl Esters and Separation of Methyl Cholate and Methyl Dehydrocholate—The crude acid precipitate was dried to constant weight at 110°, and a weighed sample was dissolved in twice its weight of a 2 per cent solution of sulfuric acid in methyl alcohol, solution being effected by gentle heating whenever necessary. The solution was chilled, then seeded with methyl cholate to induce crystallization. Methyl cholate crystallized within a short time when the acid precipitate contained 15 per cent or more cholic acid. The crystals of this ester were separated by filtration, washed with a small quantity of cold methyl alcohol, and recrystallized from anhydrous ether. The resulting product (m.p. 152–153°; $[\alpha]_D^{25} = +36^\circ \pm 1^\circ$) showed no depression in melting point when mixed with pure methyl cholate (m.p. 154–155°). When the esterifying solution did not yield methyl cholate crystals within a few hours, the mixture was allowed to stand at room temperature for several days and again chilled. If no crystallization occurred at the end of this time, water was added to 15 per cent of the original volume and the solution chilled for 24 hours. When 5 per cent or more dehydrocholic acid was present in the original precipitate, methyl dehydrocholate crystallized in clusters of needles. Such crystals were filtered, washed with 85 per cent methyl alcohol, and dried to constant weight. This compound melted at 232–234° and showed no depression in melting point when mixed with an authentic sample of methyl dehydrocholate.

Preparation and Separation of Semicarbazones—The mother liquor, remaining after removal of the bulk of methyl cholate and methyl dehydrocholate, was diluted with 20 volumes of cold water and cooled to 15°. The aqueous layer was decanted, warm water (60°) added, and the aqueous phase adjusted to pH 7.0 to 7.5 by addition of solid sodium bicarbonate. After the mixture cooled, the aqueous layer was decanted; the resinous residue was washed several times with water and dissolved in approximately 10 times its weight of methyl alcohol. This solution was then mixed with a solution¹ of semicarbazide hydrochloride and sodium acetate in methyl alcohol and heated on a bath of boiling water for 1 hour. The reaction mixture was cooled, then poured into a large volume of water, and chilled.

¹ The solution of semicarbazide hydrochloride and sodium acetate was prepared as follows: For each 10 gm. of the original crude acid precipitate, 8.2 gm. of semicarbazide hydrochloride and an equal molecular quantity of sodium acetate were dissolved in 10 cc. of water and diluted with 10 cc. of methyl alcohol.

Separation and Identification of Oxidation Products of Cholic Acid,



The resulting precipitate was filtered off, washed thoroughly with water, and dried.

The dried powder, a mixture of semicarbazones with a small amount of methyl cholate, was then extracted with anhydrous ether to remove the methyl cholate. The residue was dissolved in 4 parts of warm methyl alcohol and stored at 5° until crystallization occurred. The material separating at this point was filtered off and dried and has been designated the "disemicarbazone." The mother liquor was diluted with several volumes of water; the resulting precipitate was filtered off and dried and has been designated the "monosemicarbazone."

Preparation of 3-Hydroxy-7,12-diketocholanic Acid by Hydrolysis of Disemicarbazone—The disemicarbazone, mentioned above, was recrystallized from methyl alcohol and dried to constant weight. A 2 gm. portion of this material was added to a solution containing 10 cc. of 50 per cent methyl alcohol and 5 cc. of concentrated sulfuric acid, and the mixture refluxed for 10 minutes and then diluted with a large volume of water. The resulting precipitate was filtered off, redissolved in 10 cc. of 2 N sodium methoxide in methyl alcohol, and refluxed for 20 minutes. This reaction mixture was poured into 3 volumes of water, heated to remove alcohol, then acidified with dilute hydrochloric acid. The precipitate that formed was filtered, washed with cold water, suspended in 100 cc. of boiling water, and the water solution decanted into a beaker. Crystals formed as this solution cooled; these were purified by recrystallization from ethyl acetate, m.p. 188–189°, $[\alpha]_D^{25} = +30.8^\circ$ (alcohol). The compound contained two carbonyl groups according to analysis by the Hughes procedure and gave a negative Zimmermann reaction. When mixed with an authentic sample of 3-hydroxy-7,12-diketocholanic acid (m.p. 187–189°), there was no depression of melting point.

Reactions of Monosemicarbazone. Hydrolysis—20 gm. of the monosemicarbazone, 20 cc. of 50 per cent methyl alcohol, and 10 cc. of concentrated sulfuric acid were mixed together, heated for 30 minutes on a bath of boiling water, then diluted with 500 cc. of cold water. The resulting precipitate was filtered off, resuspended in 50 cc. of 1 N sodium hydroxide, and the mixture heated to obtain complete solution. After cooling, the solution was acidified with dilute hydrochloric acid, the resulting acid precipitate being filtered, washed with warm water, and dried.

This crude acid precipitate, $[\alpha]_D^{25} = +16^\circ$ (methyl alcohol), would not crystallize from ether, acetone, ethyl or methyl alcohol, petroleum ether, benzene, ethyl acetate, or combinations of the above solvents, or from dilute dioxane.² The compound gave negative Zimmermann and Gregory-Pascoe

² In unpublished work one of us (W. M. H.) has found that the crude acid may be crystallized from dilute acetone or dilute methanol and has been found to melt at 163–

reactions, and when treated with Girard's Reagent T according to the Hughes procedure did not form a mercuric iodide hydrazone. When the crude acid was reconverted to the methyl ester and treated with semicarbazide as described previously, a monosemicarbazone was formed (m.p. 178–180°).

Formation of Desoxycholic Acid through Wolff-Kishner Reduction of Monosemicarbazone—10 gm. of monosemicarbazone, 10 gm. of sodium, and 100 cc. of methyl alcohol were placed in a bomb and heated at 180° for 30 minutes. The resulting mixture was cooled, diluted with 50 cc. of water, and filtered. The filtrate was acidified with dilute hydrochloric acid, the precipitate formed being filtered, washed with three 100 cc. portions of boiling water, and dried (yield 8 gm.). This crude acid was crystallized from glacial acetic acid and dried at 100° (m.p. 139–140°; $[\alpha]_D^{25} = +57^\circ \pm 2^\circ$). When this compound was mixed with a sample of acetic acid-choleic acid, it showed no depression of melting point. When treated in the usual manner for conversion of acetic acid-choleic acid to desoxycholic acid, the above compound yielded desoxycholic acid (m.p. 170–173°; $[\alpha]_D^{25} = +57^\circ \pm 1^\circ$). This desoxycholic acid showed no depression in the melting point when mixed with an authentic sample of desoxycholic acid.

Formation and Reactions of Benzoxy Derivative of Monoketo Acid—The crude monoketo acid obtained from hydrolysis of the monosemicarbazone was converted to the benzoxy derivative as follows: 8 gm. were dissolved in 20 cc. of a solution of 2 per cent sulfuric acid in methyl alcohol. This solution was allowed to react for 2 hours, and was then diluted with 150 cc. of benzene and washed successively with water, 1 N sodium hydroxide, and water to remove the methyl alcohol and sulfuric acid. The benzene was removed by distillation and the residue dried *in vacuo*. This dried methyl ester was redissolved in 12.5 cc. of benzene; to this solution 3 cc. of dry pyridine and 2.4 cc. of benzoyl chloride were added. This mixture was allowed to react for 12 to 16 hours. The benzoxy derivative, separated by conventional procedures, was crystallized from dilute methyl alcohol; yield 7.1 gm., m.p. 145–146°, $[\alpha]_D^{25} = +41.5^\circ$ (methyl alcohol); saponification equivalent 255.8; C 72.94 per cent; H 8.14 per cent.³ Assuming that the compound was a monobenzoxy monohydroxy monoketocholanic acid, it should have had a theoretical saponification equivalent of 262 and should have contained 73.11 per cent C and 8.63 per cent H.

This benzoxy derivative formed a hydrazone when treated with Girard's Reagent T. 50 mg., treated according to the Hughes technique, yielded

164°; $[\alpha]_D^{25} = 0.0^\circ \pm 1^\circ$. This crystalline acid was recrystallized from ethyl acetate with excellent recovery and melted at 199–200°; $[\alpha]_D^{25} = 0.0^\circ \pm 1^\circ$.

³ These analyses were performed by the Arlington Laboratories, Chagrin Falls, Ohio.

109 mg. of mercuric iodide hydrazone. This amount was 101.3 per cent of the theoretical yield required for conversion of a methyl ester of a monobenzoxymonohydroxymonoketocholanic acid to its corresponding hydrazone.

The benzoxo derivative was converted to a semicarbazone as follows: 1 gm. of the crystalline derivative, 1 gm. of semicarbazide hydrochloride, and 0.9 gm. of sodium acetate were added to 50 cc. of methyl alcohol, and the mixture refluxed for 15 minutes, concentrated to turbidity, and diluted with water. The precipitate that formed was filtered off, dissolved in 10 cc. of methyl alcohol, and reprecipitated by the addition of water. This latter

TABLE I
Separation of Ketocholanic Acids Formed during Oxidation of Cholic Acid by
Alcaligenes faecalis

Acid ppt.	Composition of acid ppt.		Weight of sample fractionated	Cholanic acid derivatives isolated				Recovery
	Cholic acid	Derivatives of cholic acid		Cholic acid*	Monoketocholanic acid†	Diketocholanic acid‡	Triketocholanic acid§	
	per cent	per cent	gm.	per cent	per cent	per cent	per cent	per cent
A	35.3	64.7	22	34.4	65.0			99.4
B	34.6	65.4	20	35.8	64.0			99.8
C	3.3	96.7	20	2.9	78.5	5.5	13.3	100.2
D	4.5	95.5	17		35.6	23.0	4.1	62.7

* Isolated as methyl cholate.

† Isolated as methyl ester of monosemicarbazone.

‡ Isolated as methyl ester of disemicarbazone.

§ Isolated as methyl dehydrocholate.

|| Approximately half the product was lost during a transfer of the monosemicarbazone.

precipitate was dried at 110° and extracted with 50 cc. of dry ether. The resulting compound had a melting point of 178–180°. There was no depression in melting point when this material was mixed with equal quantities of the semicarbazone of the methyl ester of 3-benzoxo-12-hydroxy-7-ketocholanic acid (7).

The benzoxo derivative was oxidized with chromic acid as follows: 1 gm. was dissolved in 20 cc. of glacial acetic acid; this solution was cooled to 15°, mixed with a solution of 0.99 gm. of chromic acid in 25 cc. of 90 per cent acetic acid, allowed to react for 30 minutes, and then diluted with several volumes of water. This dilute solution was extracted with ether; the ether extract was washed with water to remove acidic and colored substances, then evaporated to dryness. The residue was crystallized from dilute

methyl alcohol, dried, and extracted with ether. The resulting compound had a melting point of 119–120° and showed no depression of melting point when mixed with an authentic sample of the methyl ester of 3-benzoxo-7,12-diketocholeic acid (8). Saponification of the above benzoxo derivative yielded 3-hydroxy-7,12-diketocholeic acid (m.p. 187–188°).

Application of Above Procedures to Digests of Cholic Acid—The above procedures have been applied to four different digests of cholic acid. According to the data presented in Table I, the monoketo acid seemed to be the sole reaction product when the acid precipitates contained about one-third cholic acid. When the precipitates contained detectable amounts but less than 5 per cent of cholic acid, the monoketo acid was still the principal product, but appreciable quantities of diketo and triketo derivatives were also present.

Comment

The preceding data suggest that 3,12-dihydroxy-7-ketocholeic acid is the first product of the oxidation of cholic acid by *Alcaligenes faecalis*. Although this compound has not been isolated as such from the digests, its presence in the digests has been proved by (1) preparation of its monosemicarbazone and reduction of this compound to desoxycholeic acid, and (2) preparation of a benzoxo derivative of the methyl ester identical with an authentic preparation of the methyl ester of 3-benzoxo-12-hydroxy-7-ketocholeic acid.

The data further suggest that 3-hydroxy-7,12-diketocholeic acid is the second product of the bacterial oxidation of cholic acid. This keto acid was separated from the crude digests as the disemicarbazone and isolated from this derivative in pure form.

These findings are of interest, for they show that *Alcaligenes* oxidized the hydroxyl at C₇ first and that at C₃ last. This order of oxidation 7 > 12 > 3 is the same as that observed by Gallagher and Long (9) in the oxidation of cholic acid by chromic acid. This order has been suggested previously by the work of Kaziro and Shimada (3) and Hoehn and Schneider (7). Haslewood (10) likewise confirms this finding.

One other observation deserves mention; namely, the failure of 3,12-dihydroxy-7-ketocholeic acid to react with Girard's Reagent T.⁴ This observation is of especial interest, for in an earlier study (2) it was noted that the amount of mercuric iodide hydrazone obtained from certain digests was only a fraction of that which should have been obtained if all

⁴ Through the courtesy of Dr. T. F. Gallagher we obtained a sample of 3,12-dihydroxy-7-ketocholeic acid prepared by oxidation of cholic acid with chromic acid. This compound, like that prepared through bacterial oxidation of cholic acid, did not form a mercuric iodide hydrazone.

the cholic acid that disappeared from the medium had been converted to a monoketo acid. This apparent discrepancy is explained by the finding that the 7-ketodihydroxy acid does not react with Girard's Reagent T. This ketocholanic acid is the only one thus far studied which does not form a Girard hydrazone.

SUMMARY

The present study was designed to determine the composition of the mono- and diketocholanic acids formed during bacterial oxidation of cholic acid to dehydrocholic acid. Methods were developed for the separation of unchanged cholic acid and its oxidation products as present in crude acid precipitates derived from bacterial digests. The methods involved esterification of cholic acid and derivatives, separation of the esters of cholic and dehydrocholic acid from those of other keto acids by fractional crystallization, formation of the semicarbazones of the mono- and diketocholanic acids, and finally separation of the mono- and disemicarbazones through fractional crystallization.

The identity of the diketo acid was established as 3-hydroxy-7,12-diketocholanic acid through preparation of this compound from the disemicarbazone.

The identity of the monoketo acid was established as 3,12-dihydroxy-7-ketocholanic acid. Its presence was proved by the fact that the acid isolated from the monosemicarbazone after conversion to the methyl ester formed a benzoyl derivative which gave a semicarbazone identical with the semicarbazone of methyl 3-benzoxy-12-hydroxy-7-ketocholanate, and that the monosemicarbazone yielded desoxycholic acid when reduced according to the Wolff-Kishner procedure.

Analysis of various digests indicated that the 7-keto acid was the first oxidation product of cholic acid, the 7,12-diketo acid being the second product. This indicates that the hydroxyls of cholic acid undergo oxidation by *Alcaligenes* in the same order as when treated with chromic acid.

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ISOLATION OF DEHYDROISOANDROSTERONE SULFATE FROM NORMAL MALE URINE*

By PAUL L. MUNSON, T. F. GALLAGHER, AND F. C. KOCH

(From the Department of Biochemistry of the University of Chicago, Chicago)

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It is well known that the steroids are excreted in urine as water-soluble substances which can be hydrolyzed with more or less ease to the unconjugated compounds, which in turn are readily soluble in a variety of organic solvents. Since free steroids are more easily fractionated by chemical methods, the established practice for investigation in this field has been to subject the urine to a hydrolytic process prior to or simultaneous with extraction by the organic solvent. Relatively little attention has been given to the combined form of urinary steroids, although it is evident from the numerous modifications suggested by various authors that the hydrolytic process is unsatisfactory. Moreover, it is clearly recognized that steroids such as chlorodehydroandrosterone, androstadienone-17, and the like are secondary products formed in the course of hydrolysis or in the isolation technique. The influence of the factor of hydrolysis on other as yet unrecognized compounds is still unknown. We have therefore undertaken an investigation of the conjugated form of the neutral 17-ketosteroid fraction of normal male urine with the aim of establishing more satisfactory methods for isolation and quantitative measurement. This end already appears to have been justified. Talbot, Ryan, and Wolfe have recently reported (1) a technique for assaying the dehydroisoandrosterone content of urine based upon our finding that this compound is excreted as the sulfate.

Conjugation with glycuronic acid is a common method by which the animal excretes water-insoluble substances. Cohen and Marrian (2) identified estriol glycuronide in pregnancy urine and Venning and Browne (3) showed that progesterone is excreted as pregnanediol glycuronide. Peterson, Hoskins, Coffman, and Koch (4) found that glycuronic acid and acid-hydrolyzable androgen obtained from normal male urine were ap-

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parently present in constant ratio through several purification procedures. Our own work provides some evidence that the neutral 17-ketosteroids may be conjugated with glycuronic acid, although we have been unable to separate a pure substance.

It is not surprising that the phenolic steroids are excreted as sulfuric acid esters; Schachter and Marrian (5) isolated an estrone sulfate from pregnant mare urine and Butenandt and Hofstetter (6) were able to show that a portion of the estrone excreted by the pregnant woman was present in urine as the sulfuric acid ester. It is, however, of considerable interest to find that a neutral steroid is excreted, at least in part, by the same method of conjugation. Venning, Hoffman, and Browne (7) have recently isolated androsterone sulfuric acid ester from the urine of a man with an interstitial cell tumor; this is an extremely significant result, since it raises the question whether all 17-ketosteroids are excreted as the sulfuric acid esters. In the present communication we shall report the isolation of dehydroisoandrosterone sulfate as the sodium salt of the water-insoluble semicarbazone from the urine of normal young men. Although our work on this problem was undertaken independently and was completed shortly after Venning, Hoffman, and Browne had identified their product, we had the privilege of examining the compound isolated by these workers and have delayed publication until after their results appeared in the literature.

EXPERIMENTAL

Determination of 17-Ketosteroid—For hydrolysis, an aliquot of aqueous or alcoholic extract containing 0.2 to 5 mg. of steroid was taken and redistilled alcohol added to a total alcohol volume of 5.0 ml. The solution was further diluted with 45 ml. of 0.5 N hydrochloric or sulfuric acid, refluxed 15 minutes, and cooled to room temperature immediately. The hydrolysate was extracted five times with 50 ml. portions of U.S.P. ether, and the combined ether extracts were washed five times with 10 per cent sodium hydroxide and five times with water, then evaporated to dryness. The residue was dissolved in redistilled alcohol, filtered, and made to volume so that the final concentration of 17-ketosteroid was 20 to 100 γ per ml.

The Holtorff and Koch (8) procedure for colorimetric estimation of 17-ketosteroids was followed with minor modifications. In the original method 0.2 ml. each of alcohol solution to be assayed, *m*-dinitrobenzene solution, and 5 M aqueous potassium hydroxide was used. In this study 1.0 ml. of steroid solution and 0.5 ml. of each of the reagents are used. The change permits assay of more dilute solutions and more accurate measurement of the sample, while the sensitivity and accuracy of the method are unimpaired. As recommended by Pincus and Pearlman (9), the final dilution was made with 60 per cent alcohol instead of 92 per cent alcohol to

eliminate the possibility of precipitation of carbonate. Robbie and Gibson (10) have recently published a similar modification.

Determination of Free 17-Ketosteroid—The content of free 17-ketosteroid was estimated in the same way, except that hydrolysis was omitted. Before extraction with ether the solution to be assayed was diluted with water instead of acid. The difference between the values for total 17-ketosteroid and free 17-ketosteroid was taken as the amount of *conjugated 17-ketosteroid*.

All the crude extracts were highly colored and some also contained substances which develop an interfering color with alkali alone. To correct for this chromogenic material, blank estimations were performed in the usual manner, except that the *m*-dinitrobenzene reagent was omitted.

Preparation and Fractionation of Urine Extracts—Urine of males was collected (under *n*-butanol in most cases) in medical school wash rooms or as pooled 24 hour specimens of graduate students, and refrigerated for a maximum of 2 to 3 days before extraction. Small urine collections were extracted with butanol (11) in 250 ml. centrifuge bottles, centrifuged, the butanol drawn off by suction, and the extraction repeated five to seven times. Larger quantities of urine, up to 144 liters, were extracted in 18 to 36 liter batches by dropping the urine into 2 liters of butanol in a 5 liter cylinder. The urine was sprayed through four 2 mm. jets terminating several cm. above the surface of the urine-butanol emulsion which developed as soon as the process was started. The aqueous phase separated in the bottom of the cylinder and was drawn off through a stop-cock for subsequent extraction through fresh butanol. Solid sodium chloride was added liberally from time to time to facilitate separation. The urine was extracted in this manner six to ten times, the time required for each extraction being about 45 minutes.

The combined butanol extracts were evaporated to dryness on the boiling water bath under diminished pressure. The residue was exhaustively extracted with 92 per cent ethanol (a total of about 2 liters for the extract from 144 liters of urine), again evaporated to dryness, and the residue extracted with water-saturated butanol in small portions totaling about 2 liters. This butanol extract was then fractionated as in the following experiment.

2.2 liters of butanol representing 144 liters of normal urine of men contained 185 gm. of solids (including some salt) and 1.03 gm. of neutral 17-ketosteroid (161 mg. of free 17-ketosteroid). This solution was chilled below 10° and washed five times with cold saturated sodium bicarbonate solution. The combined aqueous extracts contained 200 mg. of total neutral 17-ketosteroid (4.5 mg. of free 17-ketosteroid). The butanol was then thoroughly shaken with three separate 100 ml. portions of cold 2.5 N NaOH. The combined alkaline extracts contained 41.5 mg. of neutral

17-ketosteroid. It should be noted that the term "neutral 17-ketosteroid" refers to the substances determined by the color reaction after acid hydrolysis and separation of the neutral fraction without regard to the reaction of the conjugated form.

The butanol was next extracted with 100 ml. of distilled water to which had been added a small amount of sodium chloride to facilitate separation. This aqueous extract removed 7.8 mg. of neutral 17-ketosteroid. Then

TABLE I
Fractionation of Butanol Extract of 144 Liters of Male Urine

Water extract No.	Volume	0.1 N acid to neutrality, per ml. extract	17-Ketosteroid total in aqueous phase	17-Ketosteroid per ml. solvent		Ratio of butanol to water
				Aqueous phase	Butanol phase	
	<i>ml.</i>	<i>ml.</i>	<i>mg.</i>	γ	γ	
1	140	8.06	4.2			
2	149	2.74	7.9			
3	159	0.99	8.7			
4	230	0.38	11.2			
5	323	0.06	22.4			
		pH				
6	290	10	49.1			
7	335	8.6	82.4			
8	330	7.5	70.8	205	167	0.81
9	285	7.3	61.4	204	150	0.74
10	380	6.9	64.9	162	119	0.73
11	327	6.5	38.3	111	104	0.94
12	345		32.9	90	90	1.00
13	308		21.4	67	81	1.21
14	324		18.5	54	73	1.35
15	333		16.0	46	67	1.46
16	328		13.3	39	61	1.56

The total 17-ketosteroid remaining in butanol after the eighth extraction was found to be 481 mg. and after the sixteenth extraction 210 mg. The free 17-ketosteroid in the final butanol solution was 103 mg.

followed three extractions with 2.5 N NaOH which removed an additional 24.5 mg. of neutral 17-ketosteroid, an intermediate extraction with distilled water which removed but 2.3 mg. of neutral 17-ketosteroid, and a final alkaline extraction with four portions of 2.5 N NaOH which removed 18.9 mg. of neutral 17-ketosteroid. The total amount of neutral 17-ketosteroid removed from butanol by aqueous bicarbonate and alkali was 295 mg.

The butanol solution was then thoroughly extracted with four successive 150 ml. portions of 2 per cent NaCl solution and finally twelve times, each

with 350 ml. of distilled water. For the last eight extractions it was necessary to separate emulsions by centrifugation. Tables I and II show the results of this fractionation.

Water Extracts 9 to 13 were combined, extracted three times with 400 ml. portions of ether, and the aqueous solution dried in a good vacuum while frozen. The solid residue weighed 3.2 gm. This was dissolved in 25 ml. of water, 3.0 gm. of semicarbazide hydrochloride and 4.5 gm. of sodium

TABLE II

Balance Sheet for Fractionation of Butanol Extract of 144 Liters of Male Urine

Extracts	Neutral 17-ketosteroid	
	Total	Free
	mg.	mg.
Butanol extract.....	1037	137
Sum of bicarbonate, alkali, and Water Extracts 1-8.....	545	
Difference.....	492	
Found in butanol.....	481	124
Sum of Water Extracts 9-16.....	265	
Difference.....	216	
Found in butanol.....	210	103

TABLE III

Proportion of 17-Ketosteroid Precipitable by Semicarbazide in Successive Water Extractions of Butanol Extract

Water extract No.	Conjugated neutral 17-ketosteroid		
	Total	Precipitable by semicarbazide	
	mg.	mg.	per cent
Water Extract 7.....	82.4	2	2.5
“ “ 8.....	70.8	4	5.7
“ Extracts 9-13 (combined).....	218.7	114	52
“ “ 14-16 “.....	40.1	28	70
Butanol-soluble, after 16 aqueous extractions.....	107	91	85

acetate $\cdot 3\text{H}_2\text{O}$ added, and the solution warmed in a water bath at 90° for 30 minutes. After standing overnight, the precipitate was collected by centrifugation, washed twice with cold water, and dried in a good vacuum at 85° . The yellowish powder weighed 475 mg. This contained 114 mg. of 17-ketosteroid and 110 mg. were found in the mother liquors and washings. Several of the fractions were treated in similar fashion with results recorded in Table III.

Purification of Precipitate—The precipitate obtained from the combined

fourteenth, fifteenth, and sixteenth aqueous extracts was dissolved in about 40 ml. of boiling ethanol and after filtration concentrated to about 10 ml. This solution overnight deposited a bead-like precipitate weighing 16 mg., which appeared crystalline when examined with a hand lens but was amorphous under the microscope. Re-solution in ethanol and concentration of the solution yielded 4.8 mg. of solid which contained 55 per cent 17-ketosteroid.

3.291 mg. of this product yielded 0.398 mg. of white ash (which was combined with the combustion products for sulfur determination) and 1.385 mg. of BaSO_4 .¹

$\text{C}_{20}\text{H}_{30}\text{O}_5\text{N}_3\text{SNa} \cdot \text{C}_2\text{H}_5\text{OH}$. Theory. S 6.47, ash 14.33
Found. " 5.78, " 12.09

The amount of material obtained was too small for isolation. However, by the same procedure 61 mg. of an apparently identical product were obtained from the combined ninth to thirteenth aqueous extracts. This material contained 0.59 mg. of 17-ketosteroid per mg. of substance and gave a strongly positive test for sulfate. Bioassay after acid hydrolysis revealed that the product assayed 2.8 I.U. per mg. of 17-ketosteroids.² 54 mg. were hydrolyzed by boiling for 15 minutes with 55 ml. of 0.5 N HCl and the neutral fraction isolated in the usual manner. The oily residue was sublimed in a high vacuum at 78° for 24 hours. A small amount of oily material and a white crystalline sublimate formed which melted at 120–124°. ³

The residue in the retort was again sublimed at 100° and a second crystalline sublimate obtained which melted at 145–149°. The first sublimate was converted to the benzoate and a product melting at 248–250° obtained. Recrystallization from benzene-petroleum ether yielded a product melting at 253°. This melting point was unchanged by recrystallization from methanol and upon admixture with an authentic specimen of dehydroisoandrosterone benzoate (m.p. 254–255°) there was no depression. The second sublimate was converted to the benzoate and without purification melted at 237–240°. One recrystallization from benzene-petroleum ether gave a product melting at 251–252° which showed no depression when mixed with dehydroisoandrosterone benzoate. The unsublimed material in the retort was negligible in amount.

The isolation of the semicarbazone was repeated with the eighth, ninth, and tenth aqueous extractions from a separate butanol extract of normal male urine. In this case more attention was given to obtaining a satisfactorily pure sample for analysis. The total neutral 17-ketosteroid of the

¹ Microanalysis by Dr. T. S. Ma, Department of Chemistry, University of Chicago.

² Direct application to the comb; unpublished method of Johnston and Koch.

³ All melting points are corrected.

extract was 112 mg. A precipitate weighing 95.3 mg. and containing 32.1 mg. of 17-ketosteroid (34 per cent) was obtained. 62 mg. of this product were extracted with hot alcohol, leaving an insoluble residue of 19 mg. which contained less than 0.5 mg. of 17-ketosteroid. The alcohol solution deposited 26.9 mg. of precipitate which contained 11.3 mg. (42 per cent) of 17-ketosteroid. This material was twice more redissolved and reprecipitated, each time forming the characteristic bead-like aggregates noted in the foregoing experiment. The final product weighed 7.3 mg. and contained 4.42 mg. of 17-ketosteroid (60.6 per cent).

4.218 mg. of substance gave 0.592 mg. of ash and 1.942 mg. of BaSO_4 . The ash was white and completely soluble in dilute hydrochloric acid without evolution of CO_2 . Addition of BaCl_2 produced a precipitate which was combined with the products of combustion for sulfur estimation. 2.437 mg. of substance gave 0.197 ml. of nitrogen at 26° and 752 mm. The analytical figures are consistent with the semicarbazone of sodium dehydroisoandrosterone sulfate with a molecule of ethanol.

$\text{C}_{20}\text{H}_{30}\text{O}_6\text{N}_2\text{SNa} \cdot \text{C}_2\text{H}_5\text{OH}$. Theory. S 6.47, ash 14.33, N 8.47
Found. " 6.32, " 14.04, " 9.13

Recovery of Administered Dehydroisoandrosterone—Through the kind cooperation of Dr. Allan T. Kenyon of the Department of Medicine, University of Chicago, we were able to study the recovery of administered dehydroisoandrosterone. The patient (M. L.) was a short, sexually underdeveloped woman of 27 years, without proved pituitary disease. She received daily intramuscular injections of dehydroisoandrosterone acetate for 10 successive days; during the first 4 days a total of 270 mg. was administered but the urine collection for this period was not available to us. During the following 6 days a total of 600 mg. of the acetate was injected and the pooled urine collection amounted to 9.1 liters. 3.17 liters of this urine were put at our disposal; the neutral 17-ketosteroids assayed 53 mg. per liter or a total of 196 mg. This urine was extracted by shaking thoroughly with six 400 ml. portions of butanol which removed 171 mg. of neutral 17-ketosteroid (87 per cent). The combined butanol was then extracted six times with 2.5 N NaOH, with two 500 ml. portions of water, and finally with thirteen successive 200 ml. portions of water. There was a marked difference between this extract and those previously obtained from normal male urine; after these aqueous extractions 118 mg. or 69 per cent of the neutral 17-ketosteroid which was extracted from the urine by butanol still remained in the butanol phase. The butanol was removed under diminished pressure, and the residue suspended in water and extracted three times with ether. The ether removed 47 mg. of neutral 17-ketosteroid and 115 mg. of solids, which indicated that 24 per cent of the total neutral 17-ketosteroid had

been hydrolyzed in the course of fractionation. The aqueous phase contained 180 mg. of solids and assayed 65 mg. of neutral 17-ketosteroid. Therefore 52 per cent of the butanol-soluble, conjugated neutral 17-ketosteroid in this experiment was still present in the butanol phase after six alkaline and fifteen aqueous extractions, in contrast to the normal male urine in which but 10 per cent remained after similar treatment.

The aqueous solution (200 ml. volume) was warmed on the steam bath for 20 hours with 500 mg. of semicarbazide hydrochloride and 620 mg. of sodium acetate $\cdot 3\text{H}_2\text{O}$. The precipitate was collected by centrifugation, hydrolyzed by boiling for 30 minutes with 100 ml. of 0.5 N HCl, and the neutral fraction isolated as usual. The neutral fraction assayed 50 mg. of 17-ketosteroid and was directly sublimed in a high vacuum at 85° and the crystalline sublimate converted to the benzoate. 31 mg. of product were obtained which after one recrystallization from methanol melted at $249\text{--}251^\circ$ and gave no depression when mixed with an authentic specimen of dehydroisoandrosterone benzoate. The residue in the retort was again sublimed in a high vacuum and a second crystalline sublimate obtained on heating to 120° . This was likewise converted to the benzoate and 14 mg. of dehydroisoandrosterone benzoate were isolated after one crystallization from methanol. The oily residue in the retort gave no further sublimate even after being heated to 160° . The crystalline benzoate isolated accounts for 33 mg. of dehydroisoandrosterone or 25 per cent of the conjugated neutral 17-ketosteroids which were initially extracted by butanol.

Because of the pressure of other work, the aqueous extracts were not investigated.

DISCUSSION

The experiments we have reported demonstrate conclusively that dehydroisoandrosterone is excreted in normal male urine, at least in part, as an ester of sulfuric acid. In our experiments the fraction which precipitated as the semicarbazone represented 20 per cent of the conjugated neutral 17-ketosteroids (Table IV). It should, however, be borne in mind that dehydroisoandrosterone whether free or conjugated is more labile than the saturated 17-keto urinary steroids and therefore the results of isolation experiments, digitonin separations, and the like yield minimal figures. There is considerable justification, therefore, for accepting the view that this fraction consists principally of dehydroisoandrosterone. First of all we were unable to isolate any substance other than dehydroisoandrosterone. While this by itself is inconclusive, it should be noted that in the case of the woman receiving dehydroisoandrosterone parenterally a very good recovery (25 per cent of the conjugated neutral 17-ketosteroid extracted by butanol) was made from this fraction in an experiment which was in no sense quanti-

tative. Then again the experiments of Talbot, Ryan, and Wolfe (1) in which sodium dehydroisoandrosterone sulfate was added to normal urine have a bearing on this question. In the control samples, to which no 17-ketosteroid was added, their BaCl_2 hydrolytic method indicated that the β -hydroxy neutral 17-ketosteroid fraction from five pooled specimens of fresh adult urine contained from 0.0 mg. to 5.8 mg. per liter. Assuming an average value of 20 mg. of 17-ketosteroid per liter of urine (unfortunately this value was not given in their paper), the results would indicate that the β -hydroxy neutral 17-ketosteroid fraction of normal urine varied between 0.0 and 29 per cent, with an average of 14 per cent. While these calculations are subject to

TABLE IV
Quantitative Distribution of Conjugated Neutral 17-Ketosteroids

Fraction	Approximate proportion of total 17-keto- steroid	Characteristics
	<i>per cent</i>	
A*	20	Extracted from butanol by aqueous sodium bicarbonate solution
B	15	Extracted next from butanol by aqueous sodium hydroxide above pH 9
C	35	Extracted next from butanol by water below pH 9 and not pptd. by semicarbazide acetate
D	20	Extracted from butanol by water below pH 7.5 and pptd. as semicarbazone; mainly sodium salt of sulfuric acid ester of the semicarbazone of dehydroisoandrosterone
E†	10	Free, ether-soluble, 17-ketosteroid

* This fraction should probably be included in Fraction C, since the bicarbonate was neutralized by acids extracted from urine with butanol.

† This free steroid probably is liberated in the course of the treatment of large amounts of material.

error, in general they suggest a high value for the β -hydroxy-17-ketosteroid fraction and together with our experiments indicate that a considerable portion of this is dehydroisoandrosterone.

Although the amount of dehydroisoandrosterone recovered after parenteral administration in our experiments was quite large, it is questionable whether our procedure is of general applicability for pathological as well as normal urine. Since androsterone sulfate forms a water-insoluble semicarbazone (7), colorimetric assay or bioassay of this fraction unsupported by isolation of the steroid could yield extremely misleading results. This caution is, by the same token, justifiable in our figure of the amount of dehydroisoandrosterone present based on the insoluble semicarbazone fraction, for the product was purified by several precipitations before isola-

tion of the steroid was attempted. Further work by a variety of procedures is necessary before the amount of dehydroisoandrosterone normally present in urine can be satisfactorily ascertained.

The problem of hydrolysis still presents some vexing aspects. It has been repeatedly demonstrated that acid hydrolysis of urine destroys or modifies certain of the neutral steroids in one way or another. Venning, Hoffman, and Browne (7) were unable to recover a substantial portion of androsterone known to be present as the sulfate after acid hydrolysis. It should be noted that the data of these authors showed that the method of Peterson, Gallagher, and Koch (12) invariably gave a higher recovery than other procedures, although even by this method 17 to 43 per cent was lost. Similar experiments by Talbot *et al.* (1) indicate that dehydroisoandrosterone sulfate is also altered. It is possible that in our experiments the presence of the steroid as a semicarbazone had a stabilizing influence, since an intact hydroxyl group after hydrolysis was demonstrated by the formation of the benzoyl derivative.

Our results with the urine from Dr. Kenyon's patient who had received dehydroisoandrosterone acetate daily for 10 days have an interesting bearing on the metabolism of this compound. As we have indicated, our experiments were not quantitative; yet from the residual butanol after six alkaline and fifteen aqueous extractions we were able to isolate 25 per cent of the total conjugated steroid initially extracted by butanol as the crystalline benzoate. It is scarcely to be doubted that a much greater quantity of dehydroisoandrosterone could have been obtained had the late aqueous extracts and the hydrolyzed or "free" 17-ketosteroids been more carefully examined. In addition the rather large amount of "free" 17-ketosteroids found in this experiment suggests that this fraction contained a considerable amount of dehydroisoandrosterone. The conclusion is therefore inescapable that the greater portion of the compound was not metabolized further but was excreted unchanged. This is similar to the situation with administered androsterone, since Dorfman and Hamilton (13) have shown that at least 24 per cent of that substance can be recovered unaltered from the urine. It is therefore unlikely that dehydroisoandrosterone is an essential intermediate in the metabolic conversion of testosterone to androsterone (*cf.* Koch (14)).

The fractionation of the butanol extract suggests that there are three distinct classes of conjugated urinary steroids in male urine. One is readily extracted from butanol by aqueous alkali; a second is more soluble in water below pH 9 than in the more alkaline solutions and does not form a difficultly soluble derivative with semicarbazide; the third is more soluble in *n*-butanol than in water and yields a difficultly soluble semicarbazone. The quantitative distribution of these fractions is shown in Table IV. It might

be suggested that these fractions indicate different methods of conjugation and we believe this to be the fact. However, to draw a definite conclusion would be premature and we hope that further work will clarify our knowledge.

SUMMARY

Fractionation of an *n*-butanol extract of adult male urine shows that the conjugated neutral 17-ketosteroids fall into three types. One is extracted from *n*-butanol by aqueous alkali; a second is readily extracted from *n*-butanol by water below pH 9 and is not precipitated from aqueous solution as the semicarbazone; a third is more soluble in butanol than in water and forms an insoluble semicarbazone from aqueous or ethanol solution. From the last fraction a substance identified as the semicarbazone of sodium dehydroisoandrosterone sulfate was isolated. Upon acid hydrolysis this compound yielded dehydroisoandrosterone which was identified as the benzoate.

In normal urine dehydroisoandrosterone is to a considerable extent excreted as an ester of sulfuric acid.

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THE CIRCULATION OF PLASMA PHOSPHOLIPIDS: THEIR TRANSPORT TO THORACIC DUCT LYMPH*

BY W. O. REINHARDT, M. C. FISHLER, AND I. L. CHAIKOFF

(From the Divisions of Anatomy and Physiology, University of California Medical School, Berkeley)

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Two phases of the metabolism of plasma phospholipids have been dealt with recently: (1) their site of formation and (2) their rate of removal from plasma and transfer to various organs. Plasma phospholipids are formed almost exclusively in the liver (1). Although such tissues as kidney and small intestine also synthesize these compounds, they are not available to the plasma (1). The removal of phospholipids from plasma was studied by Zilversmit *et al.* 5 hours after the introduction of labeled phospholipids into the blood stream of the dog, these workers recovered 76 to 83 per cent of them in plasma, red blood cells, liver, kidney, small intestine, spleen, and muscle (2). As much as 40 to 50 per cent of the injected labeled phospholipids had, however, disappeared from the plasma. Approximately one-third of the phospholipids lost from the plasma was found in the liver.

The fate of the plasma phospholipids that enter the liver, kidney, small intestine, muscle, etc., remains to be determined. It is shown here that a portion of these phospholipids reaches the lymphatic channels. Radiophospholipids were injected into the blood stream of the dog and their recovery in lymph of the thoracic duct demonstrated. These observations show that a measurable portion of the phospholipids that leave the plasma passes through one or more tissues on its way to the lymph channels and from these is returned to the plasma.

The question as to whether phospholipids can pass a capillary membrane has been frequently considered but not settled (3). The demonstration here that labeled plasma phospholipids are rapidly transferred to thoracic duct lymph leaves no further doubt that capillary membranes or sinusoid walls are permeable to phospholipid molecules.

EXPERIMENTAL

Donor dogs received intraperitoneally an isotonic solution of Na_2HPO_4 containing from 2 to 2.5 millicuries of radiophosphorus. At a single interval thereafter (27 to 48 hours) the dogs were exsanguinated by means of a cannula introduced into the femoral artery. Approximately 1 cc. of 0.5 per cent procaine hydrochloride was used as a local anesthetic. The donor's

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blood was centrifuged for 30 minutes at 2200 R.P.M. and the plasma drawn off. This plasma, which contained radiophospholipid, was injected into the recipient.

At the time that the donor's blood was being centrifuged, the preparation of the recipient was begun. After this dog had been anesthetized by the intraperitoneal injection of 30 mg. of nembutal per kilo, the thoracic duct was exposed and a heparinized, Pyrex glass cannula inserted into it. 90 to 100 cc. of the donor's plasma were then injected into the exposed femoral vein of the recipient. In the case of Dog 1 (Table I) the collection of lymph was not begun until 135 minutes after the plasma had been injected, whereas in Dogs 2 and 3 the collection of lymph was begun before the labeled plasma was injected.

Blood obtained from the femoral vein of the recipient was centrifuged for 10 minutes at 3500 R.P.M. The plasma was removed and analyzed for phospholipid P^{32} . Lymph was centrifuged before samples of it were taken for analysis. Heparin was used as an anticoagulant for plasma and lymph.

5 cc. samples of lymph or plasma were pipetted slowly into 150 cc. of Bloor's solution (3 parts of 95 per cent ethyl alcohol and 1 part of ethyl ether) and extracted on a hot water bath at 56° for 2 hours. A second extraction with about 100 cc. of solvent was carried out for 1 hour. The extracts were filtered into Kjeldahl flasks and the residue reextracted with ethyl ether for 8 hours in a Soxhlet apparatus. The combined extracts were then concentrated *in vacuo* at 56°. The last stages of the concentration were carried out in an atmosphere of CO_2 . The concentrates (about 1 cc. of material) were then extracted with petroleum ether. The phospholipids were precipitated from aliquots of the petroleum ether extracts and their P^{32} content determined by the method of Perlman *et al.* (4).

Results

Dog 1 received intravenously 100 cc. of plasma containing a total of 759,200 counts per minute as phospholipid. The first sample of lymph was obtained during the first 36 minutes after the injection of the radiophospholipid; this sample already contained over 2000 counts per minute or 94 per cc. Lymph was collected from this dog for a total of 6 hours; the total lymph collected contained in all 28,600 counts. At the end of the 6 hours, the plasma contained 504 counts per minute per cc. If it be assumed that plasma represents 5 per cent of the body weight, the plasma still contained a total of 452,000 counts per minute at the end of this period of observation. Approximately 9 per cent of the radiophospholipid that left the plasma was recovered from the lymph collected from the thoracic duct.

By a similar calculation, it can be shown that approximately 21 per cent of the labeled phospholipid injected into the blood stream of Dog 2 was contained in the lymph recovered from the thoracic duct in 3 hours.

P^{32} was contained in the injected plasma in compounds other than phospholipids. The following calculation shows, however, that the conversion

TABLE I

Recovery of Injected Plasma Radiophospholipid in Thoracic Duct Lymph

Dog 1 received a total of 759,200 counts per minute in 100 cc. of plasma; Dog 2 received 766,000 counts per minute in 100 cc. of plasma; Dog 3 received 403,000 counts per minute in 90 cc. of plasma. Lymph samples were not collected in Dog 1 until 135 minutes after the injection of the radiophospholipid.

Dog No.	Lymph					Plasma
	Interval after injection of plasma	Total lymph collected	Rate of flow	Phospholipid P^{32} expressed as radioactive counts per min.		Phospholipid P^{32} expressed as radioactive counts per min. per cc.
				Per cc.	For total sample collected	
	min.	cc.	cc. per min.			
1 (17.9 kilos)	Before	9.9	0.49			
	4 before to					
	40 after	23.9	0.54	94	2250	
	40- 90	26.4	0.53	216	5680	937
	90-153	23.3	0.37	251	5850	806
	153-214	23.9	0.39	213	5100	
	214-244	13.4	0.45	191	2560	
	244-272	13.3	0.47	191	2540	
	272-319	13.2	0.28	167	2220	
	319-359	13.6	0.34	178	2420	504
2 (15.5 kilos)	19-10 be-					
	fore	4.9	0.54			
	5- 19	13.7	0.98	0	0	
	19- 37	14.8	0.82	50	740	
	37- 52	14.5	0.97	110	1596	1150
	52- 68	14.6	0.91	118	1723	
	68- 85	14.6	0.86	142	2073	
	85-104	14.3	0.75	140	2000	683
	104-127	14.6	0.64	137	2000	
	127-147	14.3	0.71	127	1817	
	147-171	15.5	0.65	139	2155	
	171-187	11.1	0.69	139	1542	893
	32					984
3 (8.6 kilos)	62					863
	135-137	2.0	1.0	375	750	
	160-197	8.3	0.22	215	1784	760
	197-307	9.0	0.08	295	2654	788

of non-phospholipid P^{32} present in the plasma to phospholipid P^{32} could not account for an appreciable amount of the radiophospholipid recovered from the thoracic duct lymph during the intervals studied here: dogs were

injected intravenously with inorganic phosphate containing P^{32} and lymph collected from the thoracic duct for the next 4 hours; the amount of radiophospholipid recovered per cc. of this lymph was 3×10^{-5} per cent of the injected P^{32} . In the experiments recorded in Table I approximately 400,000 to 800,000 counts per minute of phospholipid P^{32} were contained in the plasma introduced into the blood stream. At most, an equal amount of P^{32} was also present in the form of inorganic phosphate. Hence the phospholipid P^{32} formed at the expense of the inorganic P^{32} contained in the injected plasma was $(800,000 \times 3 \times 10^{-5})/100$ or 0.2 count per minute per cc. of lymph.

Comment

The rôle of thoracic duct lymph as a transport medium for phospholipid lost from plasma is shown by the total amounts of radiophospholipid recovered in the lymph. Over 20 per cent of the radiophospholipid that left the blood stream of Dog 2 in 3 hours was recovered in the lymph that drained from the thoracic duct; over 9 per cent was recovered from Dog 1 in 6 hours. Thus thoracic duct lymph serves as a medium for the return to plasma of a significant fraction of the phospholipid lost from plasma.

The rapidity by which plasma phospholipid can be transferred to thoracic duct lymph is shown by the finding of radiophospholipids in a lymph sample obtained during the first 37 minutes after the injection of labeled phospholipids into the blood stream (Dog 2). Although the passage of phospholipid molecules across a capillary or sinusoid wall can no longer be questioned, the results obtained here provide no information as to the organ or tissue in which this transfer occurs.

SUMMARY

1. A rapid transfer of plasma phospholipids to thoracic duct lymph is demonstrated. Radiophospholipid was injected into the blood stream of the dog and subsequently recovered in lymph obtained from the thoracic duct.

2. In the experiments recorded here, 9 to 20 per cent of the injected radiophospholipids that left the plasma in 3 to 6 hours appeared in the thoracic duct lymph. Approximately these amounts would have been returned to the plasma if this lymph had not been diverted.

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THE MICROBIOLOGICAL DETERMINATION OF AMINO ACIDS

I. VALINE AND ARGININE

By J. RAYMOND McMAHAN AND ESMOND E. SNELL

(From The University of Texas, Biochemical Institute, and the Clayton Foundation for Research, Austin)

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Studies by various workers (1-4) on the amino acid requirements of lactic acid bacteria have shown that these are usually complex. The development and successful use of assay methods for several vitamins based upon growth or acid production of lactic acid bacteria early suggested use of these organisms as assay agents for the determination of amino acids.

Knowledge of the non-amino acid nutrition of members of this group of bacteria has progressed to the point where it is possible to design a medium which contains essentially only substances of known chemical composition and which will support luxuriant growth of various members of the group. Early attempts to use such a medium for the estimation of a given amino acid indicated that the response to the amino acid might not be completely specific. Modification of the medium and conditions eliminated this uncertainty, however, and in this and a subsequent paper details are given for the quantitative determination of several amino acids as they occur in protein hydrolysates.

The method developed has several advantages. The laborious separations often necessary in protein analysis are eliminated. Essentially one method and one set of equipment suffice for determination of several amino acids. The method is unusually sensitive; hence the amount of protein hydrolysate necessary is very small in comparison with that necessary for analysis by more customary procedures. Analyses for a single amino acid can be carried out at several levels on the hydrolysate from less than 1 mg. of protein. The method is also applicable to a number of amino acids, such as leucine, valine, alanine, etc., for which no accurate chemical methods are at present available.

EXPERIMENTAL

Cultures and Inoculum—Stock cultures of the organisms to be used are carried as stab cultures in yeast extract-glucose-agar (1 per cent glucose, 1 per cent yeast extract, 1.5 per cent agar). Duplicate tubes of each organism used are incubated for 24 to 48 hours at 30-37°, then held in the refrigerator. One culture is reserved for use in preparing inocula from day

to day. At the end of 2 weeks, and at 2 week intervals thereafter, two more stab cultures are prepared from the unused culture; the old cultures are discarded, and the new cultures used in a similar manner in their place. *Lactobacillus casei*¹ was the principal organism used in the present investigation; *Lactobacillus arabinosus* 17-5 was used in a few experiments (Table III).

Inoculum for use in assay tubes is grown in a special medium. This medium contains per liter 5 gm. of Bacto-peptone, 1 gm. of Bacto-yeast extract, 10 gm. of sodium acetate, 10 gm. of glucose, and 5 cc. each of inorganic salt Solutions A and B.²

Of this medium, 10 cc. portions are placed in tubes which are plugged with cotton, sterilized 15 minutes at 15 pounds steam pressure, and stored until needed. 18 to 24 hours before the inoculum is to be used, a transfer is made from the stock culture to a tube of the inoculum medium, which is then incubated at 32° until used.

Basal Medium—The basal medium for the assay of amino acids is shown in Table I. All solutions were covered with a thin layer of toluene and stored in the refrigerator. Solutions 1 and 3 are stable for months; Solution 2 should be renewed at intervals of approximately 1 month. The use of two solutions for the various amino acids is arbitrary. It was found convenient to incorporate each of the amino acids not required for growth on the above medium in Solution 1, which could be made up in large amounts. Each of the amino acids found essential for growth of one or another of the organisms tested was incorporated in Solution 3. A separate Solution 3 is then made up for each amino acid to be determined, leaving out that particular amino acid, but including each of the others. Thus for work described in the present paper, two Solutions 3 were prepared, one which contained no valine, for valine assay, and one which contained no arginine, for arginine assay.

Standard Solutions—The standard solution of arginine was prepared by dissolving *l*(+)-arginine monohydrochloride in water and diluting to a volume such that 1 cc. was equivalent to 50 γ of *l*(+)-arginine (60.5 γ of *l*(+)-arginine monohydrochloride).

Owing to the difficulty involved in obtaining pure *l*(+)-valine, *dl*-valine was used as standard. Separate tests showed that *d*(-)-valine had no activity in promoting growth on the valine-free medium, while admixtures

¹ Cultures of these organisms may be secured from the American Type Culture Collection. *Lactobacillus casei* is No. 7469; *Lactobacillus arabinosus* is No. 8014.

² These solutions have the following composition: Solution A, KH_2PO_4 25 gm., K_2HPO_4 25 gm., water to make 250 cc.; Solution B, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 10 gm., NaCl 0.5 gm., $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 gm., $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.5 gm., water to make 250 cc. Solution B partly precipitates on standing; it should be shaken well before use.

TABLE I

Basal Medium for Assay of Amino Acids

Items I to V, mixed in the amounts designated, form the medium for 80 assay tubes.

I. Solution 1, 80 cc.		IV. Solution 2, 10 cc.	
Adenine sulfate.....	10 mg.	<i>p</i> -Aminobenzoic	
Guanine.....	10 "	acid.....	600 γ
Xanthine.....	10 "	Biotin.....	0.4 "
Uracil.....	10 "	Calcium panto-	
<i>l</i> (-)-Cystine.....	100 "	thenate.....	200 "
Glycine.....	100 "	Nicotinic acid....	200 "
<i>l</i> (-)-Hydroxyproline...100 "		Pyridoxine hydro-	
<i>dl</i> -Isoleucine.....	200 "	chloride.....	600 "
<i>dl</i> -Methionine.....	200 "	Riboflavin.....	200 "
<i>dl</i> -Norleucine.....	200 "	Thiamine	
<i>dl</i> -Norvaline.....	200 "	chloride.....	200 "
<i>dl</i> -Phenylalanine.....	200 "	Folic acid*.....	800 mg. units
<i>l</i> (-)-Tyrosine.....	100 "	Choline chloride..	5.0
Ammonium sulfate....	3.0 gm.	Inositol.....	5.0
Sodium acetate (an-		Water to make....	100
hydrous).....	6.0 "	V. Solution 3, 10 cc.	
Salts B.....	5.0 cc.	<i>dl</i> -Alanine.....	200 mg.
Dissolve by heating with water,		<i>l</i> (+)-Arginine	
cool, add NaOH to pH 6.8-7.0,		monohydro-	
dilute to 400 cc.		chloride†.....	100 "
II. Salts A, 2 cc.		<i>l</i> (+)-Aspartic	
III. Glucose, 2 gm.		acid.....	400 "
		<i>l</i> (+)-Glutamic	
		acid.....	1000 "
		<i>l</i> (-)-Histidine....	100 "
		<i>l</i> (-)-Leucine.....	100 "
		<i>l</i> (+)-Lysine.....	100 "
		<i>l</i> (-)-Proline.....	100 "
		<i>dl</i> -Serine.....	200 "
		<i>dl</i> -Threonine....	200 "
		<i>l</i> (-)-Trypto-	
		phane.....	100 "
		<i>dl</i> -Valine†.....	200 "
		Dissolve in 30 cc. water, add	
		NaOH to pH 6.8-7.0, dilute to	
		50 cc.	

* Any concentrate of "potency" 1000 or greater is suitable (Mitchell, Snell, and Williams (5)). Material of potency 3000 was used in the present investigation. 800 mg. units of this material weigh $800 \div 3000 = 0.27$ mg.

† When the medium is used for valine assay, valine is left out of this solution; when it is used for arginine assay, arginine is left out of the solution, etc.

of it with *l*(+)-valine had exactly the activity of the *l*(+)-valine alone. The standard solution of valine was prepared by dissolving *dl*-valine in water and diluting to a volume such that 1 cc. contained 100 γ of *l*(+)-valine (200 γ of *dl*-valine).

Procedure

Lipless, Pyrex test-tubes, 22 $\times$ 100 mm., with 2 mm. walls were found convenient and were used in the experimental work reported here. Standard size test-tubes (16 $\times$ 150 to 20 $\times$ 150 mm.) of uniform dimensions may also be used. These tubes were supported in wire racks in which they were autoclaved and incubated.

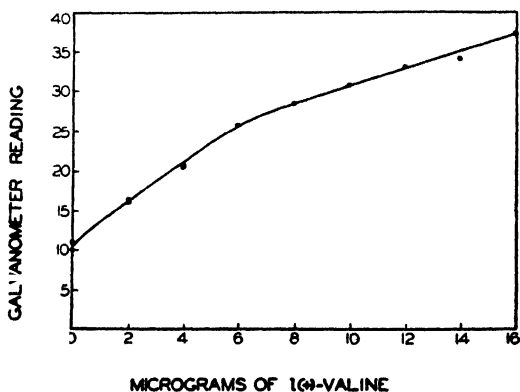


FIG. 1. Typical standard curve for assay of valine

Suitable aliquots of the standard solutions or protein hydrolysates were now added to the tubes. To establish the standard curve for valine (*cf.* Fig. 1) points at the following levels were used: 0, 0, 2.0, 2.0, 4.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, and 16.0 γ of *l*(+)-valine. With arginine, points used to establish a standard curve were 0, 0, 1.0, 1.0, 2.0, 2.0, 3, 4, 5, 6, 7, and 8 γ of *l*(+)-arginine (*cf.* Fig. 2). In each case, protein hydrolysates were taken in the quantities calculated to yield at least six points which would fall on the standard curve.

Two procedures have been commonly used. In Procedure A, the standard solutions of amino acids and the protein hydrolysates were so diluted that ordinary 1 or 2 cc. pipettes, calibrated at 0.1 cc. intervals, could be used. Total volumes up to 1.2 cc. were used; the volume in each tube was then adjusted where necessary to 1.2 cc. with water. 1.25 cc. of the appropriate basal medium, prepared as indicated in Table I, and adjusted if necessary to pH 6.8 to 7.0, were then added to each tube.

In Procedure B, the standard solutions of amino acids were used un-

diluted, and more concentrated solutions of protein hydrolysates were used, so that the total volume added with the standard or protein hydrolysate sample did not surpass 0.2 cc. For this purpose, special pipettes³ prepared from 0.2 cc. pipettes calibrated at 0.01 cc. intervals were used. When this method of introducing the sample was used, the volume of the added sample was disregarded. The basal medium prepared as indicated in Table I and adjusted if necessary to pH 6.8 to 7.0 was diluted with an equal volume of water. 2.5 cc. of the diluted medium were then added to each tube. A small amount of undissolved material occasionally present in the medium did not interfere in any way if uniformly suspended by shaking.

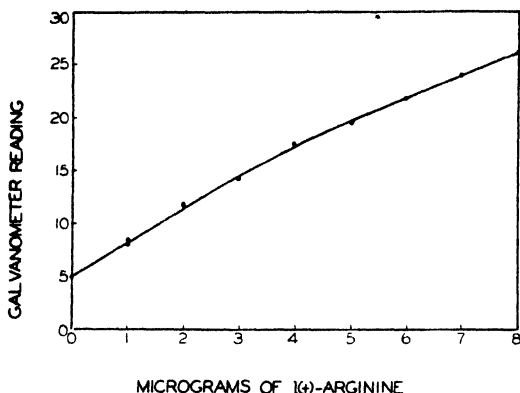


Fig. 2. Typical standard curve for assay of arginine

The assay tubes prepared by one of the above procedures were plugged with lint-free cotton in the usual manner.⁴ The racks of tubes, together with necessary pipettes or syringes, were sterilized by autoclaving at 15 pounds pressure for 15 minutes. After cooling to room temperature, the tubes were ready for inoculation.

³ A small bubble about twice the diameter of the original capillary is blown near the tip of the pipette. This is then drawn out to form a needle-like tip about 30 mm. long. The top of the pipette is then cut off about 40 mm. above the top graduation and polished. The pipettes were operated by a 1 cc. hypodermic syringe of the "insulin" type, attached to the pipette by a 50 mm. piece of pressure tubing slipped part way over the barrel of the syringe. A thin coating of light petroleum jelly on the plunger insures smooth operations. With a little practice, volumes of 0.01 to 0.2 cc. can be delivered accurately and rapidly with this equipment.

⁴ In some tests, the racks were simply covered with clean laboratory towels, folded in such a manner that each tube was covered by eight layers of cloth. Extra precautions are necessary to prevent contamination when this is done; if these are taken, trouble is rarely encountered, and the procedure is much faster than that ordinarily used.

Cells from the inoculum culture, described above, were centrifuged down. The supernatant liquid was removed, and the cells resuspended in 10 cc. of sterile 0.9 per cent sodium chloride solution. 0.2 to 0.5 cc. of this suspension was then transferred to another 10 cc. of sterile saline solution. Each assay tube was inoculated by sterile pipette or syringe (6) with 1 drop of the latter suspension. The tubes were incubated in a water bath at $32^{\circ} \pm 0.1^{\circ}$ for 72 hours.

Subsequent treatment depends upon the method chosen for measuring the growth response. If titration or pH measurements are to be used, these are carried out directly on the contents of each tube. With few exceptions, we have employed turbidity measurements. The contents of each tube were diluted with 5 cc. of water delivered from an automatic pipette. Each tube was shaken vigorously and then allowed to stand a few moments for air bubbles to rise and break. Just before being read, the tube was inverted carefully a few times, and the contents were poured into the cell of the measuring instrument. The thermocouple turbidimeter (7) was employed almost exclusively for this purpose, but trial runs with the Evelyn photoelectric colorimeter and an Aminco type F photometer showed these instruments also to be suitable; presumably any good photoelectric instrument would serve. With the turbidimeter, the galvanometer was set to read zero when distilled water was in the cell, 100 when the cell contents were completely opaque.

Samples of typical standard curves obtained are shown in Figs. 1 and 2. Similar curves must be obtained with each assay, and values for the content of a particular amino acid in the protein hydrolysate are obtained by interpolation on the corresponding standard curve. From this, the percentage of amino acid present in the original protein is calculated (*cf.* Table II). The curves for valine and arginine are usable over the entire range above the first point (2 γ for valine, 1 γ for arginine).

Preparation of Samples—The following proteins were used in this study: Labco vitamin-free casein, Knox gelatin, lactoglobulin, egg albumin, horse hemoglobin (once recrystallized), horse hemoglobin (recrystallized three times), and silk fibroin. The last five pure proteins were kindly supplied by Dr. Max Bergmann.

A preliminary survey of hydrolysis conditions necessary to liberate a maximum amount of a given amino acid from casein was made. 100 mg. of casein were placed in each of sixteen Pyrex test-tubes. To separate sets of four tubes each was added 1 cc. of 2, 5, 10, and 20 per cent hydrochloric acid. The tubes were sealed in an oxygen flame; then one tube of each acid concentration was autoclaved at 15 pounds pressure for 2, 4, 6, and 8 hours. The resultant hydrolysates were assayed microbiologically for threonine. Results with 10 and 20 per cent acid were highest, and

showed little difference at the various times. Values for the 2 per cent acid approached those given by 20 per cent acid when hydrolysis was continued for 8 hours. Later another experiment was carried out with 2 and 10 per cent hydrochloric acid and autoclaving times of 2, 4, 6, 8, 10, and 24 hours. Assays were run on each of the hydrolysates for each of the twelve amino acids listed in Solution 3, Table I, except tryptophane and proline. With most of these the values obtained after 6, 8, 10, and 24 hours of hydrolysis were almost identical whether 2 per cent or 10 per cent acid was used. The indicated valine content continued to increase slightly over long

TABLE II
Assay for Valine in Labco Vitamin-Free Casein

Assay organism, *Lactobacillus casei*.

Valine added	Galvanometer reading	Casein hydrolysate added	Galvanometer reading	Valine present	Valine present in casein
γ		γ		γ	per cent
0	10.1	40	17.9	2.75	6.9
0	10.8	60	20.2	3.75	6.3
2	16.0	60	20.8	4.00	6.7
2	16.2	80	23.2	5.25	6.6
4	20.8	80	23.0	5.15	6.4
4	20.5	100	25.9	6.70	6.7
6	24.6	100	26.3	6.95	7.0
8	28.3	120	28.1	7.90	6.6
10	30.6	120	28.0	7.85	6.5
12	32.9	140	29.7	9.10	6.5
14	34.0	140	29.7	9.10	6.5
16	37.1	160	31.2	10.4	6.5
		160	31.1	10.4	6.5
Average...					6.6

periods of time, however; and since there was apparently little destruction by prolonged time and high acid concentration, 10 per cent hydrochloric acid and 10 hours autoclaving time were selected for general use.

100 mg. of each of the above proteins were hydrolyzed by this procedure. The tubes were cooled and opened, and the hydrolysates washed out with about 95 cc. of water, adjusted with sodium hydroxide to pH 6.8 to 7.0, and diluted to a final volume of 100 cc.

The above method is well adapted to handling very small quantities of material without loss. By using small Pyrex tubes and the micro balance, quantitative assays for various amino acids have been obtained on protein samples of 1.5 to 3.0 mg.

Results of Assays—Detailed results of an assay of casein for valine are

given in Table II. The number of points included in the assay is greater than ordinarily run to give a better idea of the degree of variation encountered. A similar assay for arginine at casein levels varying arithmetically from 40 to 160 γ per tube, with use of the standard curve plotted in Fig. 2, gave percentage values for arginine in casein of 4.0, 3.8, 3.6, 3.6, 3.8, 3.4, 3.4, 3.5, 3.5, 3.5, 3.5, and 3.8, average 3.6.

The precision and probable accuracy of the test have been tested in various ways. To see how closely single points could be duplicated, a valine test was set up which contained twenty-four tubes, each containing 80 γ of casein hydrolysate. The average deviation from the mean reading was 0.4 galvanometer division. From the standard curve this deviation represented 0.19 γ of valine; since 80 γ of casein contain about 5.4 γ of valine, this represents an average deviation of less than 4 per cent of this value.

TABLE III
Recovery Experiments in Valine Assay; Comparison of Test Organisms

Assay organism	Valine found		Recovery
	Casein alone	Casein + 6.00 per cent added valine	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
<i>Lactobacillus arabinosus</i>	6.86	12.88	100
“ <i>casei</i>	6.72	12.92	103
	6.80	12.79	100

To test the reproducibility of assay values from one test to another, a series of six assays for valine was run over a period of 2 months. Different standard solutions, different pipettes, and different batches of medium were used in some of these assays, but the same casein hydrolysate was used throughout. The per cent valine found in each assay was as follows: 6.72, 6.66, 6.90, 6.46, 6.80, 6.62, average 6.69. The average deviation from the mean was 0.11 or 1.6 per cent of the mean.

Other indications of the reliability of the method may be obtained from recovery experiments, from agreement in results obtained with different test organisms, and from comparison of assay values obtained with others reported in the literature. Table III gives recovery experiments for valine with both *Lactobacillus casei* and *Lactobacillus arabinosus* as test organisms. For valine assay, there is little to choose between these two organisms. Results are in excellent agreement, and recovery of added valine is quantitative. In Table IV, recovery experiments with arginine are recorded. With both casein and gelatin, recovery of added arginine is satisfactory.

The results of assays for valine and arginine in the various proteins used are shown in Tables V and VI. Most of the values recorded in the litera-

TABLE IV

*Recovery Experiments in Arginine Assay*Assay organism, *Lactobacillus casei*.

Protein hydrolysate	Sample No.	Arginine added	Arginine found	Recovery
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Casein	I	0.00	3.93	109
		4.00	8.30	
	II	0.00	3.55	92
		4.00	7.22	
Gelatin	I	0.00	9.13	96.5
		5.00	13.95	
	II	0.00	8.55	99.5
		10.00	18.50	

TABLE V

*Microbiological Values for Valine Content of Proteins*Assay organism, *Lactobacillus casei*.

Protein	Valine content, per cent		
	Assay I	Assay II	Average
Labco casein.....			6.7*
Ovalbumin.....	6.86	6.79	6.8
Silk fibroin.....	3.11	3.31	3.2
Horse hemoglobin (once recrystallized)....	9.18	8.91	9.0
“ “ (3 times recrystallized) ..	8.86	8.77	8.8
Lactoglobulin.....	6.05	5.56	5.8
Gelatin (Knox).....	2.68	2.66	2.7

* Average of six determinations.

TABLE VI

*Microbiological Values for Arginine Content of Proteins*Assay organism, *Lactobacillus casei*.

Protein	Arginine content			Values from literature	Bibliographic reference No.
	Assay I	Assay II	Average		
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Labco casein.....	3.81	3.61	3.7	3.72	8
Ovalbumin.....	5.79	5.49	5.6	5.66, 5.6	8, 9
Silk fibroin.....	0.98	0.96	0.97	0.95, 0.76	10, 8
Horse hemoglobin (once recrystallized).....	3.47	3.53	3.5	3.59	8
Horse hemoglobin (3 times recrystallized).....	3.23	3.47	3.4	3.59	8
Lactoglobulin.....	2.92	2.90	2.9	2.89	11
Gelatin (Knox).....	9.56	9.06	9.3	9.34, 9.1	12, 9

ture for valine have been obtained by the ester method, which Vickery (13) states is unreliable. Although it is easy to find figures supporting the assay values for valine found microbiologically, these are not given, since it would be equally easy to quote figures to agree with other very different values. In the case of arginine, however, for which reliable chemical methods are available (13), results obtained by other workers are listed for comparison. Excellent agreement is evident, which indicates the reliability of the present method, not only as applied to arginine, but as applied to other amino acids as well. It was primarily to check reliability of the microbiological method that the determination of arginine was investigated.

DISCUSSION

It is well known that various lactic acid bacteria differ in their requirements for certain vitamins and growth factors. This is true also of their amino acid requirements. Thus *Lactobacillus casei* is reported to grow without glycine or alanine (4), whereas *Streptococcus lactis* R requires both of these amino acids for growth (3). The usefulness of this method for determining amino acids would thus be greatly enhanced if a medium, conditions for growth, and methods for measuring growth applicable to as large a number of organisms as possible were selected. The following features of the method were adopted in an attempt to do this: (a) incubation at 32°, (b) turbidimetric measurement of the growth response to amino acids, and (c) selection of a medium as complete as present knowledge allows. A brief consideration of each of these factors may be helpful.

While it has been customary in past methods to incubate *Lactobacillus casei* at 37°, many of the other organisms of use in microbiological methods for amino acids (*Lactobacillus arabinosus*, *Lactobacillus pentosus*, *Leuconostoc mesenteroides*, *Streptococcus lactis*) have a temperature optimum nearer 30°. In practice, all of these organisms grow well at temperatures intermediate between the two extremes. It is more practicable to maintain one incubator at a temperature at which all of the organisms grow well rather than to maintain a number of different incubators. If equipment is available, however, there is no objection to the higher temperature (37°) for *L. casei*. Thus two parallel assays of casein, with *L. casei* as the test organism, one incubated at 32°, the other at 37°, yielded values for valine of 6.58 and 6.60 per cent, respectively, and the standard curves were almost exactly superimposable.

Turbidimetric measurement of the growth response was selected because it is more rapid than titration and is applicable to organisms such as *Streptococcus lactis* and *Leuconostoc mesenteroides* which do not produce sufficient acid during growth for convenient titration. While it may be argued that

measurement of protoplasmic mass (turbidity), presumably directly dependent upon the concentration of an essential amino acid, is more logical than measurement of a more indirect function, such as acid production, in practice the two methods give identical results when acid production can be used. Thus acid titration (electrometric) of an assay with *Lactobacillus casei* on casein yielded an average value for valine of 6.63 per cent. Agreement between values obtained from duplicate tubes and at various assay levels was excellent.

Many of the amino acids present in the medium are not essential for growth of the test organisms. Growth of these organisms upon an otherwise adequate medium containing only the indispensable amino acids is not as heavy as when complete amino acid mixtures are used (4). Mixtures containing each of the well recognized amino acids, on the other hand, support growth as well as do hydrolysates of complete proteins, such as casein. Antagonistic effects between amino acids, which occur frequently on media containing restricted mixtures of amino acids (4, 14), occur much less frequently when each of the known amino acids is used in fairly substantial quantity. The possibility that such antagonisms may occur must, however, be borne in mind when the method is applied to new amino acids. Asparagine, known to stimulate growth of *Lactobacillus casei* and other lactic acid bacteria (15, 16), was not added to the medium. Its effect is duplicated by glutamic acid (15) which is present in large amounts. Its addition does not alter assay results, but for certain purposes, such as assay of glutamic acid, application of the assay to enzymatic protein digests, or to hydrolysates of unpurified natural materials, its addition is advisable.

In the case of valine, and all other cases so far tested, the assay organisms have been able to use only the naturally occurring, *l* form of the amino acid. *dl*-Amino acids can be used in the basal medium interchangeably with natural amino acids, but when thus used they are added in double the quantity used for the natural acid. In application of the method with a new organism, or to a different amino acid, the configurational specificity of the test organism to the amino acid should be determined if possible error is to be avoided.

Of the vitamins and growth factors included in the basal medium only choline and inositol have not been shown to be essential for some organism of the lactic group. A number of them (*e.g.* pyridoxine, riboflavin, thiamine, folic acid) could be dispensed with if one of the less fastidious organisms, such as *Lactobacillus arabinosus*, was used exclusively for assay.

The effect of incubation time has been studied briefly. After a 3 day incubation period there is no tendency of values to "drift" upward or downward at increasing dosage levels. When the incubation period is shortened

to 24 hours, growth has not proceeded to completion and such drift becomes evident, higher values being obtained at the low levels.

In general, the values obtained are in somewhat better agreement with each other than when similar tests are used for vitamin assay. This is to be expected, since the substances to be determined constitute a larger proportion of the sample, and the sample itself does not contain so many materials likely to interfere with the assay. Preliminary attempts to apply the assay to hydrolysates of crude protein-containing materials, such as dried egg, dried pork, etc., indicate that the method may also be useful here.

Slightly lower blanks are obtained if cells for inoculum are grown in the amino acid basal medium with a minimal amount of the amino acid to be determined. Assays secured with such inocula tend to be somewhat erratic, however. Much more regular results are obtained when the inoculum is grown in the special enriched medium recommended above. This probably indicates stimulative action by small amounts of a substance or substances not furnished in the basal medium, but carried over in adequate amounts by cells of the inoculum when these are grown in rich media. Kuiken *et al.* (17) in a preliminary note have indicated requirement for such a stimulative substance by *Lactobacillus arabinosus* when this organism is used for amino acid assay in a basal medium based on that of Snell and Wright (18). These authors also found isoleucine to be an essential amino acid for *Lactobacillus arabinosus*, a finding which did not appear from the above work. This discrepancy is most probably due to contamination of some of the natural amino acids used in the medium with minute amounts of isoleucine. Thus, with an essential amino acid required in the same amount as is arginine, a cumulative impurity of this amino acid amounting to only 0.14 per cent of the natural amino acids added per tube would be sufficient to furnish all of it required, and thus completely mask the requirement for it. Contamination of the natural amino acids with traces of other amino acids is the chief factor contributing to the high blanks observed in the assay of some amino acids. Obviously, the best way to circumvent this situation is to replace the natural amino acids of the medium by synthetic products whenever these are available.

The use of large quantities of amino acids in basal media for amino acid assay is expensive. Partly for this reason, the assay was carried out upon a 2.5 cc. scale rather than upon the more customary 10 cc. scale. The precision of the assay is not increased in any way by using the larger volume, while the "micro" nature of the assay is influenced adversely; but there is no objection to using the larger volume, especially if acid titration is used to follow growth. Since growth on the medium is quite heavy, dilution is advisable preliminary to making turbidity measurements, and the smaller volume is more convenient here.

A number of applications for the general method outlined in the present paper suggest themselves. Besides being applicable where more customary methods serve, its sensitivity should permit application to determination of the amino acid composition of rarer proteins, for which data are extremely scarce at present. When the unnatural form of an amino acid is inactive, the method can be used to determine the rate of racemization as influenced by the presence of other optically active compounds. Whether amino acids are fully available to the organisms when combined in soluble peptides has not been determined; if so, analyses could be carried out on enzymatic digests of proteins.

SUMMARY .

A medium and procedure are outlined for the quantitative determination of valine and arginine in protein hydrolysates, based upon their essential nature for *Lactobacillus casei* and *Lactobacillus arabinosus*. It is accurate, applicable to the detection of minute amounts of amino acids, and requires no extensive pretreatment of the protein hydrolysate. By suitable modifications of the basal medium, and the use of a number of organisms, the procedure is applicable to determination of several amino acids besides valine and arginine. Possible variations and applications of the technique are briefly discussed.

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DETERMINATION OF THE IODINE NUMBER OF WHOLE PHOSPHOLIPID

By P. L. MacLACHLAN

(From the Department of Biochemistry, School of Medicine, West Virginia University, Morgantown)

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Direct determination of the iodine number of whole phospholipid, as described by Yasuda (1), has been found to give erratic and variable results (Boyd (2)). This has been confirmed by the author, and is apparently due to the presence of magnesium chloride in the chloroform solution of the phospholipid. Reliable and reproducible iodine numbers were obtained when the chloroform solution of phospholipid was evaporated to dryness and the phospholipid redissolved in chloroform prior to carrying out the determination.

Boyd's solution to this problem was to determine the iodine number of the phospholipid fatty acids following saponification and extraction with petroleum ether. However, it has been shown by Wilson and Hansen (3) that the phospholipid fraction of human blood serum contains a variable amount of unsaponifiable phospholipid, which averages about 19 per cent (range, 4 to 32 per cent) of the total phospholipid. This observation has been found by the author to be true for blood plasma and other tissues as well (Table I). Therefore, the use of the saponification procedure may introduce an appreciable error into the iodine number of phospholipid fatty acids.

The use of moist ether to dissolve the phospholipid, as described by Bloor (4), followed by evaporation and re-solution in chloroform, has been found to give fairly reliable iodine numbers for whole phospholipid. However, Kirk, Page, and Van Slyke (5), and more recently Sinclair and Dolan (6), have shown that some of the phospholipid from both blood and tissues does not redissolve in moist ether. The amount of ether-insoluble phospholipid was found to depend upon the amount of magnesium chloride used as well as upon the source of the phospholipid. All other conditions being constant, the percentage of ether-insoluble phospholipid was higher for blood plasma than for various other tissues.

EXPERIMENTAL

When chloroform was used to dissolve the phospholipid, as described in the procedure of Yasuda (1), unreliable iodine numbers were obtained. As will be shown later, reliable and reproducible iodine numbers were obtained when a 1:1 mixture of chloroform and ether was used, instead of

chloroform, as a solvent for the phospholipid. Experiments showed, however, that the chloroform-ether mixture did not dissolve the phospholipid as completely (92 per cent) as did chloroform (Table II). It was found, moreover, that 2:1 or 3:1 mixtures of chloroform and ether did not dissolve the phospholipid more completely than did a 1:1 mixture.

Direct determination of the iodine number on a chloroform solution of phospholipid frequently resulted in very low titration values (0.02 to 0.15

TABLE I

Unaponifiable Phospholipid Content of Blood and Various Tissues, As Percentage of Total Phospholipid

The figures in parentheses represent the range of values.

Source of phospholipid	No. of samples	Unaponifiable phospholipid
Blood plasma (dog).....	4	21.6 (18.5-24.2)
Red blood cells (dog).....	4	45.0 (43.4-47.8)
Smooth muscle (pigeon gizzard).....	4	28.7 (19.6-42.0)
“ “ (rabbit uterus).....	4	38.2 (20.3-55.5)
Skeletal muscle (rat).....	8	5.5 (2.4-12.5)
Heart muscle (pigeon).....	1	9.2
Tumor tissue (rat).....	6	4.1 (3.0- 5.2)

TABLE II

Data on Egg Yolk Phospholipid and Magnesium Chloride with Chloroform and Chloroform-Ether Mixtures As Solvents

Solvent	No. of samples	Phospholipid	Phospholipid recovered*	MgCl ₂ taken up
		mg.	per cent	mg.
Chloroform.....	6	2.85 ± 0.01		4.6 ± 0.0†
Chloroform-ether (1:1).....	6	2.63 ± 0.02	92.2 ± 0.8	1.3 ± 0.0
“ (2:1).....	2	2.63 ± 0.03	92.4 ± 1.2	1.4 ± 0.1
“ (3:1).....	2	2.65 ± 0.02	92.9 ± 0.8	1.2 ± 0.1

* Expressed as per cent of the value obtained with chloroform.

† Average of duplicate determinations.

cc. of 0.02 N Na₂S₂O₃) for the subsequent determination, indicating little or no excess bromine. In order to ascertain whether the magnesium chloride was the factor responsible, an experiment was carried out to determine whether any magnesium chloride was taken up when chloroform was used to dissolve the phospholipid. Samples of phospholipid were precipitated with acetone and 0.1 cc. of a 30 per cent solution of magnesium chloride in 95 per cent alcohol (27.6 ± 0.3 mg.) and the precipitated phospholipid dissolved, either chloroform or chloroform-ether mixtures being used.

Determination of the residual magnesium chloride in the undissolved residue, by the Volhard-Harvey method (7), showed that more magnesium chloride was taken up when chloroform was used to dissolve the phospholipid than when a mixture of chloroform and ether was used (Table II). Apparently the chloroform solution of phospholipid tends to hold appreciable amounts of magnesium chloride; in fact the amount of magnesium chloride taken up (4.6 mg.) exceeded the phospholipid in amount (2.85 mg.) when chloroform alone was used as the solvent. The presence of ether in the chloroform lessened this tendency appreciably, and equally well in ratios of 1:1, 2:1, or 3:1 of chloroform and ether.

That the solvent *per se* was not responsible for taking up the magnesium chloride was shown by the finding that blank determinations, with chloroform, chloroform-ether (1:1), or moist ether as the solvents, gave complete recovery of the magnesium chloride in the undissolved residue. With 27.6 ± 0.3 mg. of magnesium chloride, the amounts recovered were, respectively, 27.9 ± 0.3 , 27.7 ± 0.1 , and 27.5 ± 0.0 mg.

In an attempt to explain the unreliable iodine numbers for phospholipid obtained when chloroform was used as the solvent, it was shown that the presence of magnesium chloride in the pyridine sulfate dibromide reagent alone was not responsible. Addition of varying amounts of magnesium chloride (0.0 to 13.8 mg.) to blank determinations did not affect the titration values obtained; the average titration value obtained for eight determinations was 3.60 ± 0.01 cc. of $0.02\text{ N Na}_2\text{S}_2\text{O}_3$. It was found, moreover, that the addition of varying amounts of magnesium chloride (0.0 to 16.6 mg.) to samples of egg yolk phospholipid dissolved in chloroform was without effect on the iodine numbers obtained; the average iodine number obtained for twelve samples was 81.0 ± 0.7 .

Recent work in this laboratory on the degree of unsaturation of the blood lipids of fasted mice (unpublished) gave very variable and unreliable iodine numbers for the phospholipid fraction; the iodine numbers obtained for fourteen samples ranged from 92 to 723. Four samples of egg yolk phospholipid, precipitated with acetone and magnesium chloride and dissolved in chloroform, gave very high and variable iodine numbers (216 ± 42) (Table III). In contrast, both chloroform-ether and moist ether solutions of the same phospholipid gave reliable iodine numbers. Since the latter solvents necessitate evaporation to dryness and re-solution of the phospholipid in chloroform prior to carrying out the determination, it was suggested that the process of evaporation to dryness might be responsible for the improved values. To test this, seven samples of egg yolk phospholipid were precipitated with acetone containing 0.1 cc. of a 30 per cent solution of magnesium chloride in 95 per cent alcohol, and the precipitated phospholipid was dissolved in chloroform. Two aliquots of each sample were

taken and one of each pair evaporated to dryness and redissolved in chloroform. The average iodine number obtained for the seven samples which had been subjected to evaporation was 75.8 ± 0.9 , which is in good agreement with the values obtained for egg yolk phospholipid with either chloroform-ether mixture or moist ether as the solvent (Table III). Determination of the iodine number of the seven samples which had not been evaporated to dryness showed little or no excess bromine; the titration values amounted to 0.0 to 0.02 cc. of 0.02 N $\text{Na}_2\text{S}_2\text{O}_3$, indicating very high iodine numbers.

TABLE III
Data on Phospholipid from Different Sources

Chloroform, chloroform-ether (1:1), and moist ether were used as solvents. 0.1 cc. of 30 per cent magnesium chloride in 95 per cent alcohol (27.6 ± 0.3 mg.) was used.

Source of phospholipid	Solvent	No. of samples	Phospholipid	Phospholipid recovered*	Iodine No.
			mg.	per cent	
Egg yolk	Chloroform	4	2.38 ± 0.02		216.0 ± 42.0
	Chloroform-ether	4	2.18 ± 0.01	91.6	76.0 ± 0.0
	Moist ether	2	2.10 ± 0.02	88.2	76.5 ± 0.5
			mg. per cent		
Whole blood (mouse) " " (human)	Chloroform-ether	6	328 ± 0		87.7 ± 0.4
	Chloroform†	6, A	308 ± 4		80.8 ± 0.2
	Chloroform-ether	2, "	295 ± 2	95.7	80.8 ± 0.2
	Moist ether	2, "	288 ± 4	93.5	79.5 ± 0.5
	Chloroform-ether	6, B	302 ± 0		88.5 ± 0.7
	Moist ether	6, "	293 ± 0		86.3 ± 1.1

* Expressed as per cent of the value obtained with chloroform.

† Average of duplicate determinations.

‡ Subjected to evaporation for determination of the iodine number.

The chloroform solution of the phospholipid samples which had been subjected to evaporation invariably appeared cloudy, whereas the original chloroform solution was clear. It is suggested that during the precipitation of phospholipid with acetone and magnesium chloride the latter tends to form a complex with the phospholipid which is soluble, to some extent, in chloroform and is responsible for the unreliable iodine numbers obtained. Evaporation to dryness may alter this complex so that it does not interfere in the subsequent determination of the iodine number.

Chloroform proved to be the best solvent for phospholipid. With the oxidative method of Bloor (4), the phospholipid values obtained when a chloroform-ether mixture and moist ether were used as the solvents were

approximately 92 and 88 per cent, respectively, of that obtained when chloroform was used (Table III).

In the procedure finally adopted 7 cc. of acetone and 0.1 cc. of 30 per cent solution of magnesium chloride in 95 per cent alcohol were used for the precipitation of the phospholipid, as recommended by Boyd (8) and Sinclair and Dolan (6), followed by solution of the phospholipid with chloroform, in two successive portions of 7 and 3 cc. each. After centrifugation a suitable aliquot of the chloroform solution of the phospholipid was evaporated to dryness and the phospholipid redissolved in chloroform for the determination of the iodine number. Results of analyses of phospholipid from blood, with chloroform (subjected to evaporation), chloroform-ether (1:1), and moist ether as the solvents, are given in Table III.

Six samples of phospholipid from mouse whole blood gave uniform iodine numbers when a chloroform-ether mixture was used as the solvent and showed approximately the same degree of unsaturation as phospholipid from human blood.

The iodine numbers obtained for the phospholipid from each of two samples, A and B, of human blood were very uniform when chloroform (subjected to evaporation), chloroform-ether (1:1), or moist ether was used as the solvent (Table III). When the phospholipid fatty acids are computed as two-thirds of the phospholipid, the average iodine number (83) agrees with the value (124) reported by Boyd (2) for human blood plasma. The value obtained for the phospholipid content of the blood when chloroform was used as the solvent was higher than those obtained when a chloroform-ether mixture and moist ether were used. In this respect chloroform is the solvent of choice.

SUMMARY

The use of chloroform as a solvent for phospholipid, precipitated with acetone and magnesium chloride, results in very erratic and unreliable iodine numbers. This apparently is due to a tendency of the chloroform solution of phospholipid to hold appreciable amounts of magnesium chloride.

Reliable and reproducible iodine numbers were obtained when the chloroform solution of the phospholipid was evaporated to dryness and the phospholipid redissolved in chloroform prior to carrying out the determination. Reliable iodine numbers for phospholipid were also obtained when chloroform-ether (1:1) and moist ether were employed as solvents.

The chloroform-ether mixture proved to be a better solvent than moist ether for phospholipid; however, neither of these solvents dissolved the phospholipid as completely as did chloroform.

The author wishes to express his appreciation to Mr. Robert C. Meade for technical assistance.

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SYNTHESIS OF A SECOND ISOMERIC FORM OF 3,4-DIAMINO-TETRAHYDROTHIOPHENE

BY GLEN W. KILMER AND HERBERT McKENNIS, JR.

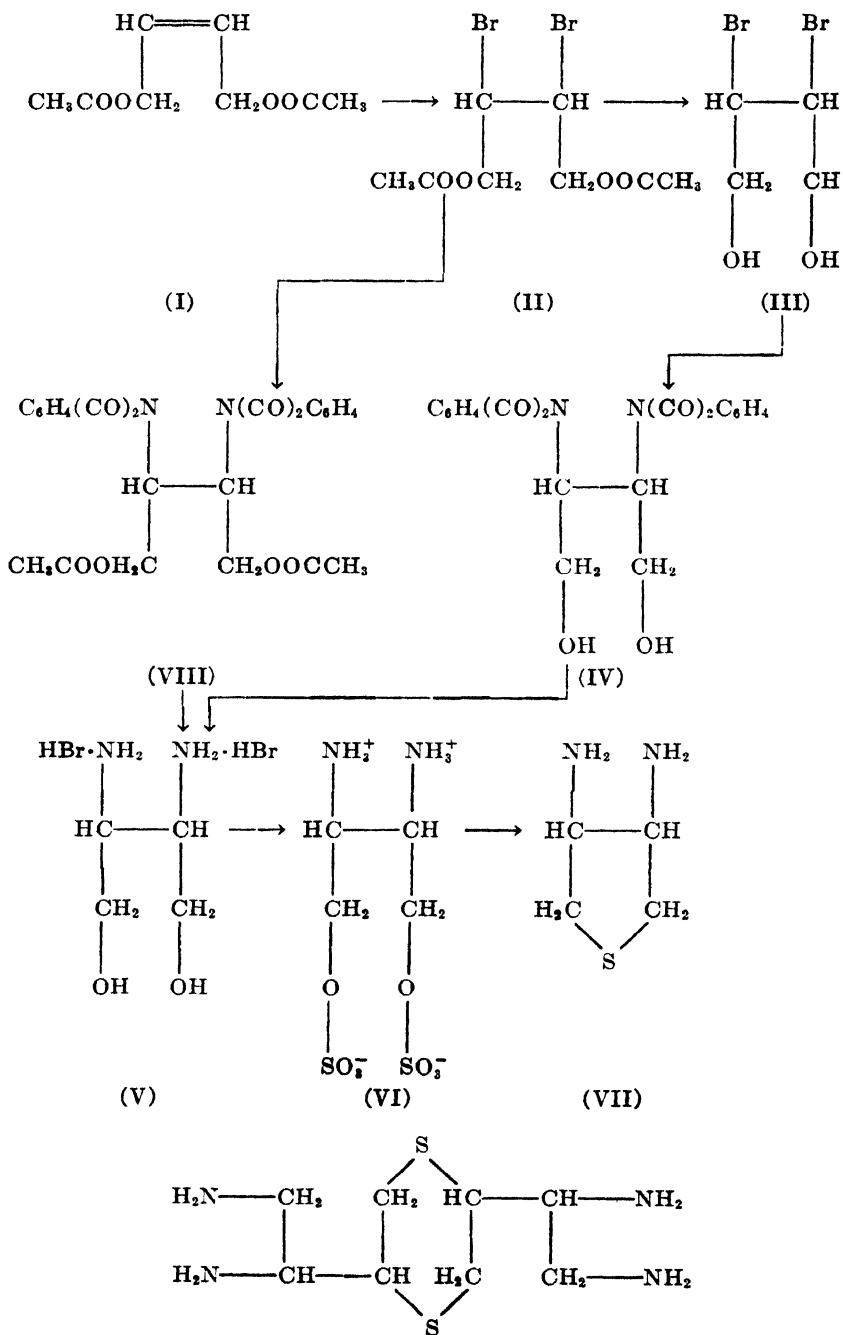
(From the Department of Biochemistry, Cornell University Medical College, New York City)

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In a recent communication from this laboratory (1) the synthesis of a 3,4-diaminotetrahydrothiophene and a comparison of its properties with those of the diaminocarboxylic acid derived from biotin were reported. The present report deals with the synthesis of the diastereoisomeric 3,4-diaminotetrahydrothiophene, which for convenience is designated Isomer B. The steps in the synthesis are shown in the accompanying reactions.

1,4-Diacetoxybutene-2 (I), prepared from 1,4-dibromobutene-2 and potassium acetate (2-4), was used as starting material in the present synthesis. Addition of bromine to (I) gave the dibromide (II) which was briefly described by Griner (2). Methanolysis of (II) or, much less expediently, hydrolysis of (II) with dilute acid produced 2,3-dibromobutanediol-1,4 (III) which Prévost (4) prepared by the addition of bromine to 2-butenediol-1,4. Compound (III) was converted to the bis(phthalimido) derivative (IV), which was hydrolyzed to 2,3-diaminobutanediol-1,4 dihydrobromide (V). This compound was also prepared by conversion of the diacetoxydibromobutane to 1,4-diacetoxy-2,3-bis(phthalimido)butane (VIII), which was then hydrolyzed to compound (V). The latter route was found to be less advantageous. Further evidence that these different routes led to the same steric form of compound (V) was afforded by a comparison of the dibenzoyl derivatives of the samples prepared by each method.

Attempts to replace the hydroxyl groups of (IV) by halogens by use of either thionyl chloride, phosphorus pentachloride, or phosphorus and iodine, were not very promising. Also the preparation of the 1,4-dihalo-butane derivative from (V) or its dibenzoyl or bis(*p*-bromobenzenesulfonyl) derivatives by use of a variety of reagents was unsatisfactory. In contrast to ethanolamine, which gives a good yield of bromoethylamine hydrobromide when treated with fuming hydrobromic acid (5), compound (V) or (VIII) when similarly treated gave only a 1 to 2 per cent yield of 1,4-dibromo-2,3-diaminobutane. The latter compound was isolated as the dibenzoyl derivative. Compound (V) failed to react with phosphorus pentachloride in the presence of acetyl chloride under the conditions used to replace the hydroxyl group of serine (6). Also when (V) was refluxed



for 2 to 4 hours in 57 per cent hydriodic acid solution, over 90 per cent of the starting material could be recovered.

However, conversion of the diaminodiol (V) to 3,4-diaminobutane-1,4-disulfuric acid (VI) proceeded very smoothly. The latter compound upon treatment with sodium sulfide gave a 3,4-diaminotetrahydrothiophene (Isomer B) (VII) which was isolated as the dipicrate. The compound was further characterized as the crystalline dibenzoyl derivative, which melted at 238–239°. The dibenzoyl derivative of Isomer A melted at 295–300°. The diacetyl derivative of Isomer A sublimed at about 260–265° (1), whereas that of Isomer B melted at 173–175°. Isomer B appeared to react with phenanthrenequinone under mild conditions, but all efforts to isolate a dibenzoquinoxaline derivative for comparison with that reported for Isomer A were unsuccessful.

Both of the isomeric 3,4-diaminotetrahydrothiophenes, when heated with phosphoric acid, evolved vapors which gave the indophenine reaction for thiophene with isatin and sulfuric acid. Furthermore when these vapors in each case were collected in carbon tetrachloride and the solution was treated with bromine (7), tetrabromothiophene was isolated.

If an allylic rearrangement¹ had occurred in the preparation of 1,4-diacetoxybutene-2 (I), it is not inconceivable that the end-product of the synthesis could have a bimolecular structure of the type shown by formula (IX). However, this structure would not be expected to yield thiophene when heated with phosphoric acid. In addition a determination of the molecular weight of the dibenzoyl derivative agreed within experimental error with that expected for the dibenzoyl derivative of the monomeric compound (VII).

Whereas biotin can be resynthesized (8) from the diaminocarboxylic acid derived from it by treatment with phosgene and sodium hydroxide, it was reported that under these conditions Isomer A did not yield a cyclic urea derivative (1). Likewise no cyclic urea was isolated after Isomer B was treated with phosgene and sodium hydroxide. It is apparent that structural features in addition to the configuration of the amino groups are responsible for the ease of formation of biotin from its diaminocarboxylic acid, which has been shown to be 3,4-diamino-2-tetrahydrothiophene-*n*-valeric acid (8–10).

EXPERIMENTAL

1,4-Diacetoxy-2,3-dibromobutane—This compound was prepared by Griner (2) but since few details were given our procedure is given in full. 80 gm. of 1,4-diacetoxybutene-2 (2, 3) were dissolved in 382 cc. of dry

¹ Prévost (4) stated that the diacetoxybutene obtained from 1,4-dibromobutene-2 was a 1,4 derivative, but did not present the evidence that no allylic rearrangement had occurred.

chloroform, the solution was cooled to between -30° and -40° , and 74.5 gm. of bromine in 410 cc. of dry chloroform were added dropwise with stirring during 4.5 hours. After standing for 3 to 5 hours at -30° , the solution was removed from the dry ice bath and was decolorized with dilute sodium thiosulfate solution. The chloroform layer was separated, was washed with water, and was distilled *in vacuo* without drying. The oily solid left as a residue was drained on a filter and washed with a 1:2 mixture of ether and hexane. More oil was removed on filter paper. 125 gm. of the dibromo derivative were obtained. The yield represents 81 per cent of the theoretical yield. The melting point was $83.5-86.5^{\circ}$ (capillary). Griner reported a melting point of 87° . Purification of our crude product for analysis was effected by precipitating the compound from chloroform by addition of hexane and subsequent recrystallization from benzene. The micro melting point of the purified material was $84-85^{\circ}$.²

$C_8H_{12}O_4Br_2$ (331.9). Calculated, C 28.95, H 3.62; found, C 29.02, H 3.98

2,3-Dibromobutanediol-1,4—A solution of 904 mg. of 1,4-diacetoxy-2,3-dibromobutane, 3.6 cc. of methanol, and 0.01 cc. of concentrated HCl was allowed to stand at $45-47^{\circ}$ for 26 hours (11). The solution was concentrated to dryness *in vacuo*; the residue consisted of almost colorless prisms, m.p. $131-131.5^{\circ}$ (capillary). The yield was almost quantitative. A sample was purified for analysis without improvement of the melting point by successive recrystallizations from water and alcohol. Prévost (4) prepared this compound in a different way but reported few data other than the melting point ($132.5-133^{\circ}$, corrected).

$C_4H_8O_2Br_2$ (247.9). Calculated, C 19.38, H 3.25; found, C 19.56, H 3.50

2,3-Bis(phthalimido)butanediol-1,4—2.6 gm. of 2,3-dibromobutanediol-1,4 and 15.3 gm. of potassium phthalimide were finely powdered and to the mixture 100 cc. of dry xylene were added. The mixture, protected from atmospheric moisture by a calcium chloride tube, was refluxed at a bath temperature of 170° for 73 hours (12). Caked material was crushed at frequent intervals during the heating. The cooled reaction mixture was filtered and the solid material was washed thoroughly with 5 per cent KOH and then with water. The dried insoluble material weighed 1.44 gm., which represents 36 per cent of the theoretical yield. The product melted at $295-299^{\circ}$. This compound is very sparingly soluble in the usual solvents except 1,4-dioxane and ethylene glycol monoethyl ether. Successive recrystallization from these latter solvents yielded colorless crystals, m.p. $300-302^{\circ}$.

² All melting points, unless otherwise noted, were taken on a micro melting point apparatus.

$C_{20}H_{16}O_6N_2$ (380.4). Calculated, N 7.37; found, N 7.36

2,3-Diaminobutanediol-1,4 Dihydrobromide—400 mg. of the diphthalimidodiols and 14 cc. of 48 per cent HBr were refluxed for 21 hours. The mixture was cooled with stirring and was then filtered. Phthalic acid was washed from the product with absolute ethanol and ether. The residue weighed 274 mg., which represents 92 per cent of the theoretical yield. The salt was purified for analysis by solution in a small volume of water and precipitation with dioxane. The pure material consisted of striated plates which began to decompose at about 220°.

$C_4H_{14}O_2N_2Br_2$ (282.0). Calculated, N 9.94; found, N 10.09

Dibenzoyl-2,3-diaminobutanediol-1,4—By treatment of the diamine with excess benzoyl chloride and 10 per cent NaOH a dibenzoyl derivative which was insoluble in dilute acid could be obtained. The compound was crystallized from absolute alcohol and from dioxane; the white flaky prisms melted at 208–209°.

$C_{18}H_{20}O_4N_2$ (328.4). Calculated, N 8.55; found, N 8.32

Tetrabenzoyl-2,3-diaminobutanediol-1,4—In some experiments it was possible to isolate from the mother liquors of the dibenzoyl derivative a tetrabenzoyl derivative. The compound crystallized from absolute alcohol as long prisms, m.p. 203–204°.

$C_{32}H_{28}O_8N_2$ (536.6). Calculated, N 5.22; found, N 5.45

Bis(p-bromobenzenesulfonyl)-2,3-diaminobutanediol-1,4—Treatment of a sample of the amine salt with *p*-bromobenzenesulfonyl chloride and alkali by a procedure similar to that used by Adams and Cairns (13) for ethanolamine yielded an acid-insoluble alkali-soluble bis(*p*-bromobenzenesulfonyl) derivative. After repeated recrystallization from alcohol and from acetone the compound was obtained as colorless crystals, m.p. 245–247°.

$C_{16}H_{18}O_6N_2Br_2S_2$ (558.2). Calculated, N 5.02; found, N 5.36

1,4-Diacetoxy-2,3-bis(phthalimido)butane—4.7 gm. of 1,4-diacetoxy-2,3-dibromobutane and 21 gm. of finely powdered potassium phthalimide were refluxed in 45 cc. of dry xylene for 41 hours. The solid material was collected on a filter and was washed by suspension in hot benzene, followed by cold 5 per cent NaOH and finally water. The white solid remaining weighed 1.2 gm., which corresponds to 19 per cent of the theoretical yield; the melting point was 238–240°. The material could be purified by recrystallization from dioxane or acetic acid. The pure material formed white granular crystals, m.p. 250°.

$C_{24}H_{20}O_8N_2$ (464.4). Calculated, N 6.03; found, N 5.79

When this compound was heated for 10 hours with boiling 48 per cent HBr as described above for the bis(phthalimido)diol, 2,3-diaminobutanediol-1,4 dihydrobromide was obtained in good yield. That this route yielded the same compound as that described above was shown by preparation of the dibenzoyl derivative from the salt prepared by hydrolysis of the diacetoxybis(phthalimido) compound. This derivative had the same melting point as that of the dibenzoyl derivative described above and an equal mixture of the two showed no depression in the melting point.

Upon evaporation the xylene-benzene liquors from the bis(phthalimido) derivative yielded a halogen-containing compound. After several crystallizations from alcohol the material formed beautiful white blades, m.p. 148–149°. The nitrogen content checked with that for the monophthalimido derivative 1,4-diacetoxy-2-bromo-3-phthalimidobutane. The yield of crude monophthalimido derivative was about 20 per cent of the theoretical yield based on the diacetoxydibromo compound.

$C_{16}H_{16}O_6NBr$ (398.2). Calculated, N 3.52; found, N 3.42

1,4-Dibromo-2,3-bis(benzamido)butane—1.0 gm. of diacetoxybis(phthalimido)butane and 20 cc. of HBr (saturated at 0°) were heated for 80 minutes at 170° in a sealed tube (5). The reaction mixture was concentrated slightly, was cooled, and the phthalic acid was collected on a sintered glass filter. The filtrate was then concentrated to a volume of about 2 cc., small quantities of solid were removed by filtration, and the oil was treated in ice-cold NaOH solution with benzoyl chloride. The solid material was recrystallized several times from alcohol-water and formed long colorless prisms, m.p. 156–157°. The yield was 13 mg. or about 1.3 per cent of the theoretical yield.

$C_{18}H_{18}O_2N_2Br_2$ (454.2). Calculated, Br 35.19; found, Br 35.21

2,3-Diaminobutane-1,4-disulfuric Acid—880 mg. of the dioldiamine dihydrobromide were dissolved in 7.8 cc. of water. The solution was cooled in ice and 975 mg. of pure silver sulfate were added slowly with stirring. The AgBr was collected on a filter and was washed, and the filtrate was treated with decolorizing carbon, was refiltered, and was evaporated to dryness *in vacuo*. To the crystalline sulfate were added about 610 mg. of concentrated sulfuric acid (14), and the mixture was heated in a bath at 140° for 25 minutes with stirring. The product was cooled in ice and 5 cc. of water were added, followed by enough 10 per cent KOH for solution of the solid. The filtered solution was acidified to Congo red with dilute HCl and the white precipitate was collected and was washed with water. The material weighed 874 mg., which is approximately the theoretical yield. A sample was purified by several reprecipitations from alkaline solution with

dilute acid. The pure material decomposed from 280° on the micro melting point stage.

$C_6H_{12}O_8N_2S_2$ (280.3). Calculated, N 10.00; found, N 9.97

3,4-Diaminotetrahydrothiophene Dipicrate (Isomer B)—450 mg. of the disulfuric acid ester, 960 mg. of $Na_2S \cdot 9H_2O$, and 40 cc. of water were heated for 3 hours at 140° in a sealed tube (15). The solution was made quite strongly acid, was treated with decolorizing carbon, and was filtered. The filtrate was concentrated to a volume of about 3 cc. This solution was refiltered, was diluted to 35 cc., and 730 mg. of picric acid were added. The solution was heated to boiling with stirring. After several hours in a refrigerator the yellow solid was collected on a filter and was washed with water. Recrystallization of the material from hot 50 per cent alcohol after treatment with decolorizing carbon gave 230 mg. of the dipicrate, representing 25 per cent of the theoretical yield. The compound crystallized as pale yellow prisms. When heated, the compound started to decompose at 220°.

$C_{16}H_{14}O_{14}N_8S$ (576.4). Calculated, S 5.56; found, S 5.19

3,4-Bis(benzamido)tetrahydrothiophene (Isomer B)—The diamino compound could also be isolated from the reaction mixture after treatment with benzoyl chloride and alkali as the dibenzoyl derivative. The compound crystallized from alcohol as colorless prisms, m.p. 238–239°.

$C_{18}H_{16}O_2N_2S$. Calculated. C 66.23, H 5.56, N 8.58, S 9.82
(326.4) Found. " 66.29, " 5.59, " 8.41, " 9.60

The experimental values for the molecular weight (Rast camphor method) were 338 and 332.

3,4-Bis(acetamido)tetrahydrothiophene (Isomer B)—300 mg. of 3,4-diaminotetrahydrothiophene dipicrate were suspended in 10 cc. of 15 per cent HCl. After extraction with benzene until free of picric acid, the aqueous solution was evaporated to dryness under reduced pressure. The syrupy residue was dissolved in 2 cc. of water and was treated with excess acetic anhydride and 10 per cent NaOH in the usual fashion. The reaction mixture was made acid to litmus by addition of 10 per cent HCl. This mixture was dried in the frozen state under diminished pressure, and the residue was extracted with chloroform. Evaporation of the chloroform solution yielded a glass which was sublimed at 150° and 1 mm. pressure. The sublimate crystallized from acetone as long white prisms melting at 135–141°. After they were dried at 100° under 1 mm. pressure, the melting point was 173–175°.

$C_8H_{14}O_2N_2S$ (202.3). Calculated, N 13.85, S 15.82; found, N 13.89, S 15.41

Isolation of Tetrabromothiophene after Decomposition of Diaminotetrahydrothiophene—40 mg. of the dipicrate of 3,4-diaminotetrahydrothiophene (Isomer B) were converted to the dihydrochloride as described above, and the oily residue left after evaporation of the water and excess HCl was treated with 1 drop of 85 per cent H_3PO_4 . The mixture was gradually heated to 400° and was maintained at this temperature for 15 minutes. The evolved vapors were collected in 1 cc. of ice-cold carbon tetrachloride. A drop of this solution gave a strong green color with isatin- H_2SO_4 . The main portion of the solution was treated with 3 drops of bromine. The flask was stoppered and was allowed to stand for about 20 hours. The solution was evaporated under diminished pressure, and the residue was dissolved in fresh carbon tetrachloride. The solution was dried over magnesium sulfate and was again evaporated. To the residue were added 2 drops of bromine. As the bromine evaporated, the product solidified (7). After purification by two recrystallizations from alcohol-water about 1.4 mg. of long colorless prisms were obtained. These were identified as tetrabromothiophene by melting point and mixed melting point. Tetrabromothiophene was likewise isolated when Isomer A of 3,4-diaminotetrahydrothiophene (1) was similarly treated.

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SUMMARY

Treatment of a 2,3-diaminobutane-1,4-disulfuric acid (prepared by a series of standard reactions) with aqueous sodium sulfide at 140° resulted in the formation of the cyclic thio ether, 3,4-diaminotetrahydrothiophene. This compound (Isomer B) differed from the 3,4-diaminotetrahydrothiophene (Isomer A) which was previously described (1).

Isomer B differed from A not only in its derivatives but also by its failure to yield a dibenzoquinoxaline derivative. It was similar to Isomer A in its failure to yield a cyclic urea under the conditions which lead to the resynthesis of biotin from 3,4-diamino-2-tetrahydrothiophene-*n*-valeric acid.

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THE MECHANISM OF KETOL FORMATION FROM PYRUVATE AND ALDEHYDES

By ROBERT L. BERG AND W. W. WESTERFELD

(From the Department of Biological Chemistry, Harvard Medical School,

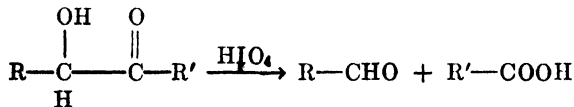
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It was previously observed (1) that the animal carboxylase enzyme system that converts pyruvate to acetoin can utilize acetaldehyde in this reaction. Other aldehydes are also utilized in the formation of analogous ketols, and the reaction between pyruvate and propionaldehyde results in a 5-carbon ketol according to Reaction I or II. The exact location of the carbonyl and hydroxyl groups in the final product has an important bearing



on the mechanism of the reaction, since in this compound the origin of each group can be established. The product is obviously formed by condensation of the 2-carbon fragment of decarboxylated pyruvate with the 3-carbon propionaldehyde, and the identification of the substituents with either the 2-carbon or the 3-carbon portion of the molecule will define the origin of each.

The identification of this compound was made by periodate oxidation. Studies showed that related compounds possessing adjacent hydroxyl or carbonyl groups were quantitatively split by periodate oxidation; the hydroxyl group was converted to an aldehyde, and the carbonyl was oxidized to an acid according to the general equation



Application of this oxidation to the 5-carbon ketol produced in the enzymatic reaction between pyruvate and propionaldehyde resulted in the formation of propionaldehyde and acetic acid. The structure of the original product therefore is given in Reaction I. Since the carbonyl group in the ketol was attached to the 2-carbon portion of the structure, it was derived from the decarboxylated pyruvate and was probably the carbonyl group originally present in the pyruvate. Similarly the hydroxyl group carried by the 3-carbon portion of the molecule must have been derived from the aldehyde group of propionaldehyde.

Identification of this structure casts serious doubt on the possibility of compound formation between pyruvate and propionaldehyde prior to the decarboxylation, since the α -carbon atom in pyruvic acid would necessarily be involved in any such structure (as it is in the final product), and it already has its four valences substituted in pyruvic acid. Intermediate compound formation could take place prior to decarboxylation only through utilization of one of the carbon bonds attached to the carbonyl group of pyruvic acid, and, if this had taken place, the hydroxyl group of the final product would have been derived from the pyruvate portion of the structure rather than from the aldehyde portion.

EXPERIMENTAL

The 2,3-butylene glycol, acetoin, and diacetyl were obtained¹ in a relatively pure condition, and were used without further purification. The ketol produced in the enzymatic reaction between pyruvate and propionaldehyde was prepared as follows: Fresh, trimmed pig heart was minced twice in a meat grinder and thoroughly washed for several hours with cold running water. 375 gm. of the pressed out mince were homogenized in a Waring blender with 550 cc. of 0.2 M phosphate buffer, pH 7.0. 15 cc. of 0.5 per cent MgSO_4 , 20 cc. of 0.1 per cent diphosphothiamine, 25 cc. of 1.0 M pyruvic acid neutralized with an equivalent amount of Na_2CO_3 , and 50 cc. of 1.0 M propionaldehyde were added, and the mixture was incubated at 37° for 5½ hours. The solution was then deproteinized with tungstic acid and the filtrate neutralized and distilled. Excess propionaldehyde was removed from the distillate by heating and aerating until no more aldehyde was carried over in the aeration gas and the solution gave no precipitate with 2,4-dinitrophenylhydrazine at room temperature. The solution was then neutralized to phenolphthalein with a small amount of NaOH , and again distilled, the first portion of distillate being discarded. The final distillate gave the 2,4-dinitrophenylosazone derivative previously described (1) for the 5-carbon ketol, and was essentially free of interfering substances as determined by the equivalent amounts of aldehyde and acid produced in the periodate oxidation.

Periodate Oxidation—The periodate oxidation was carried out by gently refluxing for 2 hours a solution composed of 100 cc. of 0.3 per cent KIO_4 , 500 cc. of water containing the test substance, and 10 cc. of concentrated H_2SO_4 (2). The aldehyde produced in the reaction was removed by continuous aeration into bisulfite, and it was estimated quantitatively by the usual iodometric method for determining bound bisulfite (3). At the end of 2 hours, the reaction mixture was distilled under standard conditions until 500 cc. of distillate were collected, and the volatile acid contained

¹ Lucidol Corporation, Buffalo, New York.

therein was titrated to phenolphthalein with standard NaOH. The total amount of acid produced in the reaction was obtained from a calibration curve prepared by a similar distillation of known amounts of acetic acid (4).

The results obtained by periodate oxidation of acetoin, 2,3-butylene glycol, diacetyl, and the 5-carbon ketol produced in the enzymatic reaction between pyruvate and propionaldehyde are summarized in Table I. The splitting of these compounds is essentially quantitative; the glycol gave rise to 2 moles of aldehyde, diacetyl gave 2 moles of acid, while each of the ketols gave 1 mole of aldehyde and 1 mole of acid. Periodate oxidation of these model compounds possessing only one pair of adjacent hydroxyl or carbonyl groups, therefore, led to a rupture of the bond between the 2 substituted

TABLE I
Periodate Oxidation of Simple Substances Possessing Hydroxyl or Carbonyl Substituents on Adjacent Carbon Atoms

Substance tested	Amount oxidized	Aldehyde formed	Acid formed	Aldehyde identified	Acid identified
	<i>mm</i>	<i>mm</i>	<i>mm</i>		
2,3-Butylene glycol	0.522	0.986	0.065	Acetaldehyde	Acetic
	0.522	0.976	0.022		
	0.522	0.981	0.035		
Acetoin	0.667	0.646	0.638	“	
	0.667	0.658	0.714		
	0.667	0.605	0.692		
Diacetyl	0.388	0.004	0.750	“	
	0.388	0.007	0.749		
	0.388	0.007	0.781		
3-Hydroxy- <i>n</i> -penta- none-2*		0.522	0.523	Propionaldehyde	“
		0.353	0.368		

* Product of enzymatic reaction between pyruvate and propionaldehyde.

carbon atoms; substituent hydroxyl groups were converted to aldehydes, while carbonyl groups were converted to acids.

Identification of Aldehydes—The aldehydes formed by periodate oxidation were identified as 2,4-dinitrophenylhydrazone derivatives. The derivative was prepared after the bisulfite titration was completed by adding HCl to a concentration of 2 N and an excess of 2,4-dinitrophenylhydrazine dissolved in 2 N HCl. The hydrazone precipitated rapidly at room temperature, and was filtered, washed thoroughly with water, and recrystallized from hot 95 per cent alcohol.

The aldehyde produced by periodate oxidation of 2,3-butylene glycol and acetoin was identified as acetaldehyde. The melting points² of the 2,4-

² All melting points are uncorrected.

dinitrophenylhydrazone derivatives were 161° and 160° respectively, and neither showed a depression when the substance was mixed with the 2,4-dinitrophenylhydrazone of acetaldehyde (m.p. 162°).

The aldehyde produced by the oxidation of the ketol formed in the enzymatic reaction between pyruvate and propionaldehyde was identified as propionaldehyde. The 2,4-dinitrophenylhydrazone derivative after several recrystallizations melted at 146°; on being mixed with the 2,4-dinitrophenylhydrazone of propionaldehyde (m.p. 149°), it melted at 148°. It had the orange color of the propionaldehyde derivative, rather than the yellow color of the acetaldehyde derivative. A colorimetric determination (5) of the amount of acetaldehyde produced in the oxidation of this ketol showed that less than 0.5 per cent of the bisulfite-binding material formed was acetaldehyde; the reaction product, therefore, contained no significant amount of acetoin or the 5-carbon isomer represented in Reaction II.

Identification of Acids—The acid portion of the split-product was identified as the *p*-phenylphenacyl ester. The sodium salt formed in several titrations was concentrated by evaporation and treated with *p*-phenylphenacyl bromide according to the directions given by Drake and Bronitsky (6). After the material was decolorized with norit and recrystallized several times from hot ethyl alcohol to constant melting point, the esters of the acids formed by periodate splitting of acetoin, diacetyl, and the 5-carbon ketol formed enzymatically all melted at 110.5°. There was no depression of melting point when each of the esters was mixed with the *p*-phenylphenacyl ester of acetic acid (m.p. 110.5°); all three showed a depression of 18–20° when mixed with the *p*-phenylphenacyl ester of propionic acid (m.p. 102°).

DISCUSSION

The product formed by the addition of benzaldehyde to fermenting yeast was identified by Neuberg and Ohle (7) as phenylacetylcarbinol, and the implications of this fact with respect to the mechanism of ketol formation were later discussed by Dirscherl (8). The ketol produced in the reaction between pyruvate and propionaldehyde in the presence of the animal carboxylase system is analogous to the product obtained by Neuberg and Ohle, since in both compounds the carbonyl group is associated with that portion of the ketol structure which is derived from pyruvate. This is incompatible with the formation of an intermediate compound between the aldehyde and the α -keto acid prior to decarboxylation of the latter, except through the postulation of a secondary reversal in the location of the carbonyl and hydroxyl groups in the ketol after it has been formed (9). The optical activity of the final products (10) makes this reversal improbable.

Ketol formation from pyruvate or from pyruvate and an aldehyde does not involve the formation of molecular acetaldehyde from the pyruvate with subsequent coupling of 2 molecules of aldehyde, for the following reasons. The condensation of acetaldehyde to acetoin (in the absence of pyruvate) is much slower than the formation of acetoin from pyruvate alone (1). Such a reaction would not account for the increased rate of decarboxylation of pyruvate in the presence of an aldehyde (1). If α -ketobutyric acid were first decarboxylated to molecular propionaldehyde, there would be no formation of propionin in the reaction, since propionaldehyde alone is not condensed by the enzyme (1).

Any mechanism for the formation of ketols from pyruvate and an aldehyde, therefore, must involve the utilization of "nascent" acetaldehyde, since (a) decarboxylation of the α -keto acid apparently precedes condensation with the aldehyde, and (b) the decarboxylated product is not molecular acetaldehyde. Furthermore, the condensation actually involves a molecule of "nascent" acetaldehyde formed in the decarboxylation of pyruvate with a molecule of the added aldehyde. Only by such a postulation could the 5-carbon ketols be formed in the reactions between pyruvate and propionaldehyde and between α -ketobutyric acid and acetaldehyde (1). In the formation of acetoin from pyruvic acid alone, it is possible that the 1st molecule of pyruvate is decarboxylated with formation of molecular acetaldehyde, and that the latter then reacts with a mole of "nascent" acetaldehyde produced by the decarboxylation of a 2nd molecule of pyruvate.

SUMMARY

Periodate oxidation of simple compounds related to acetoin and possessing adjacent hydroxyl or carbonyl groups leads to a rupture of the bond between the 2 substituted carbon atoms and a conversion of the substituent hydroxyl groups to aldehydes, while the carbonyl groups are converted to acids.

Periodate oxidation of the 5-carbon ketol produced in the enzymatic reaction between pyruvate and propionaldehyde resulted in the formation of propionaldehyde and acetic acid, thereby identifying the ketol as acetyl-ethylcarbinol (3-hydroxy-*n*-pentanone-2).

Association of the carbonyl group of the ketol with the 2-carbon portion of the structure derived from pyruvate makes doubtful the possibility of intermediate compound formation between the propionaldehyde and pyruvate prior to decarboxylation of the latter.

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GLYCINE OXIDASE*

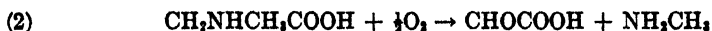
By S. RATNER, V. NOCITO, AND D. E. GREEN

(From the Departments of Medicine and Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

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The general impression that the metabolism of glycine is unique among the amino acids arises perhaps as much from the lack of specific knowledge of the immediate breakdown products of glycine as from the multiplicity of reactions in which the glycine molecule participates. The older evidence bearing on the formation from glycine of products such as NH_3 , glyoxylic acid, urea, and glucose has been indirect and controversial (*cf.* Dakin (1) and Bach (2)). Recent studies with isotopes have clarified some of these issues. Thus, Olsen, Hemingway, and Nier (3) showed the rapid formation of C^{13}O_2 from C^{13} -labeled glycine, while Ratner, Rittenberg, Keston, and Schoenheimer (4), with the aid of N^{15} -labeled glycine, showed the utilization of the amino group for synthesis of urea and other amino acids. In striking contrast, however, experiments with tissue slices and *brei* have made practically no contribution thus far to the problems of whether glycine is broken down and by what pathways. In fact, the apparent resistance of glycine to oxidation under the conditions of experiments with tissue slices has led Bach (2) to postulate that deamination of glycine follows only after preliminary condensation with some keto acid. The present communication has some bearing on the metabolism of glycine in that it deals with the properties of a widely distributed enzyme of animal origin which catalyzes the oxidation of glycine.

The enzyme which we shall refer to as glycine oxidase is a flavoprotein which can be resolved under acid conditions into a protein and flavin adenine dinucleotide (FAD), neither of which is active alone. Full catalytic activity can be restored at neutral or alkaline pH when the specific protein and an excess of the coenzyme are brought together. The enzyme catalyzes the aerobic oxidation of both glycine and sarcosine according to the following equations:



Glyoxylic acid is formed by both the deamination of glycine and the demethylamination of sarcosine.

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Glycine oxidase has been found in the liver or kidney of all animals tested. Pig kidney has proved to be a satisfactory source for routine preparation of the enzyme. Full details of the method are given in the experimental section. The final preparation contains other enzymes in addition to glycine oxidase, in particular *D*-amino acid oxidase and catalase. Splitting of the enzyme takes place during the isolation procedure. Hence it is necessary to add FAD in order to get maximum catalytic activity.

Glycine oxidase reacts with molecular oxygen, methylene blue, and other suitable hydrogen acceptors. The reaction velocity with oxygen as acceptor is linear for the first hours. The time-activity relationship is shown in Fig. 1. There is no difference in the rate of oxygen consumption whether

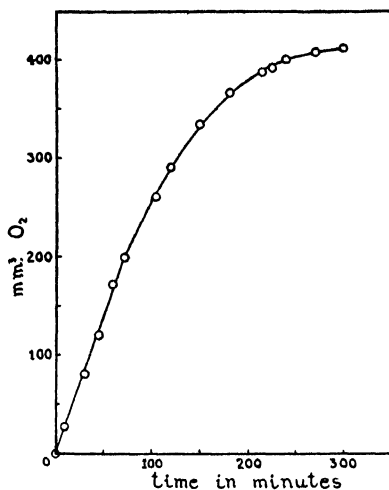


FIG. 1. Time-activity curve of glycine oxidase of pig kidney. The manometer cups contained 0.5 cc. of *m* glycine, 0.1 cc. of 10 mg. per cent flavin adenine dinucleotide, and 1 cc. of enzyme. Final volume 3.1 cc.; 38°; alkali in center well.

the gas space in the manometer contains air or pure oxygen. Addition of methylene blue does not increase the rate of oxygen consumption. This indicates that the autoxidation of the reduced enzyme is not the limiting factor in the speed of the over-all reaction.

The activity of the enzyme has also been followed anaerobically in Thunberg tubes with methylene blue as the indicator. Under constant experimental conditions, the rate of methylene blue reduction is directly proportional to the concentration of glycine enzyme. Both the manometric and Thunberg methods of estimating enzyme activity have been used interchangeably in the present investigation.

According to equations (1) and (2), for each atom of oxygen absorbed 1 molecule of substrate is oxidized with formation of 1 molecule of glyoxylic acid and 1 molecule either of NH_3 or methylamine. The data shown in Table I are in quite good agreement with the requirements of the equations. The 2,4-dinitrophenylhydrazone of glyoxylic acid was isolated in good yield after contact of the enzyme system with both glycine and sarcosine. The nitrogenous oxidation product of sarcosine, *viz.* methylamine, was also isolated and identified in the form of its picrolonate.

Flavoproteins as a class produce H_2O_2 when reacting with molecular oxygen. It follows therefore that 1 molecule of substrate should react with 1 molecule of oxygen in contrast to the 1 atom of oxygen postulated in equations (1) and (2). Such indeed would be the case if H_2O_2 accumulated in the reaction. Since an atom of oxygen is formed from H_2O_2 by action of

TABLE I

Relationship between Oxygen Uptake and Formation of Keto Acid and Volatile Base during Oxidation of Glycine and Sarcosine

The manometer cups contained 1 cc. of enzyme solution, 0.1 cc. of flavin adenine dinucleotide solution (107 γ per cc.), 0.5 cc. of 0.5 M dimethylglycine buffer, pH 8.3, 0.5 cc. of M substrate, and 0.2 cc. of 6 N NaOH in the center well. The final volume was made up to 3 cc. with water. After temperature equilibration the substrate was tipped in from the side arm. All values are corrected for enzyme blanks.

Substrate	Time	Oxygen uptake	NH_3 or CH_3NH_2	Keto acid
	<i>min.</i>	<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>
Glycine.....	130	33.7	33.3	33.6
Sarcosine.....	130	16.6	17.8	16.6

catalase which is present in excess in preparations of glycine oxidase, the net reaction involves only 1 atom of oxygen per molecule of glycine.

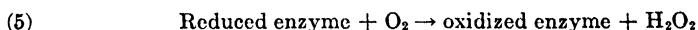
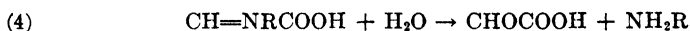
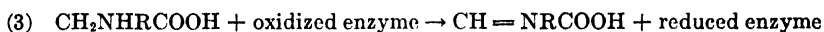
Enzyme Specificity—In addition to glycine oxidase, three other enzymes have been isolated which deaminate amino acids oxidatively; *viz.*, the *d*-amino acid oxidase (5), the *l*-glutamic dehydrogenase (6), and the *l*-amino acid oxidase (7). The identity of the glycine enzyme with the last two can be excluded, since preparations of the glycine enzyme are readily made which do not attack either *l*-glutamic acid or any of the *l*-amino acids. However, our preparations of the glycine enzyme invariably contain *d*-amino acid oxidase. That they are two distinct enzymes is supported by the following considerations. (1) The glycine enzyme may be completely split under conditions¹ which do not appreciably split the *d* enzyme

¹ For details of the conditions for splitting, see the section "Reversible splitting."

and (2) the reconstitution of the split glycine enzyme does not proceed quantitatively under conditions which permit quantitative reconstitution of the split *d* enzyme.

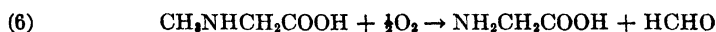
The glycine enzyme catalyzes the oxidation of glycine and N-monomethylglycine but not that of N-dimethylglycine. Substitution products of glycine such as phenylglycine, *p*-aminophenylglycine, creatine, and hippuric acid are not attacked and peptides of glycine such as glycyl-*p*-aminobenzoylglycine, glycylglycine, and leucylglycylglycine are attacked immeasurably slowly if at all.

Mechanism of Oxidation—The available data are consistent with the following mechanism of the oxidation of glycine (where R is H) and sarcosine (where R is CH₃).



Since N-monomethylglycine but not N-dimethylglycine can be attacked, the primary reaction may be considered to be the dehydrogenation of the amino acid to the imino acid. The hydrolysis to the keto acid is generally presumed to be a spontaneous process. The above mechanism is identical with the one suggested by Krebs (5) for the enzymic oxidation of the *d*-amino acids and by Richter for the oxidation of amines (8).

Handler, Bernheim, and Klein (9) found that broken cell suspensions of liver tissue will oxidize sarcosine to form glycine and formaldehyde, as shown in equation (6).



This reaction may be viewed as a dehydrogenation similar to that postulated for other amino acids, except that a hydrogen atom from the methyl group would be involved rather than 1 from the α -carbon atom. This would lead to the formation of the hypothetical intermediate $\text{CH}_2=\text{N}-\text{CH}_2-\text{COOH}$ from which glycine and formaldehyde would result. Thus the presence of the N-methyl group in sarcosine offers for the removal of 2 hydrogen atoms two theoretical possibilities, both of which have been realized enzymically.

Properties of Enzyme—The glycine enzyme works optimally only in the presence of comparatively large concentrations of substrate ($> \text{M}/6$). The half speed concentration is about 0.04 M (*cf.* Fig. 2).

The pH-activity curve is shown in Fig. 3. The maximum pH is about 8.3. The rates fall off very sharply to either side of the maximum. Somewhat surprising is the fact that at pH 7.0 the velocity is only about one-eighth of the maximum. Veronal buffers inhibited the action of the

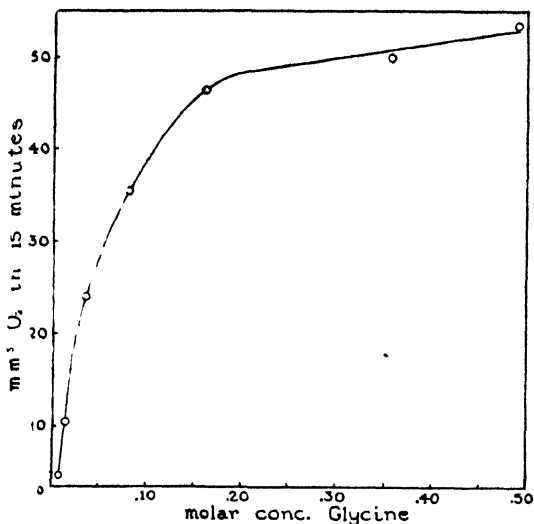


FIG. 2. Rate of oxygen uptake as a function of the concentration of glycine. Each manometer cup contained 1 cc. of enzyme, 0.5 cc. of 0.5 M dimethylglycine buffer, pH 8.3, and 0.1 cc. of 10 mg. per cent flavin adenine dinucleotide. Final volume 3.0 cc.; 38°; alkali in center well.

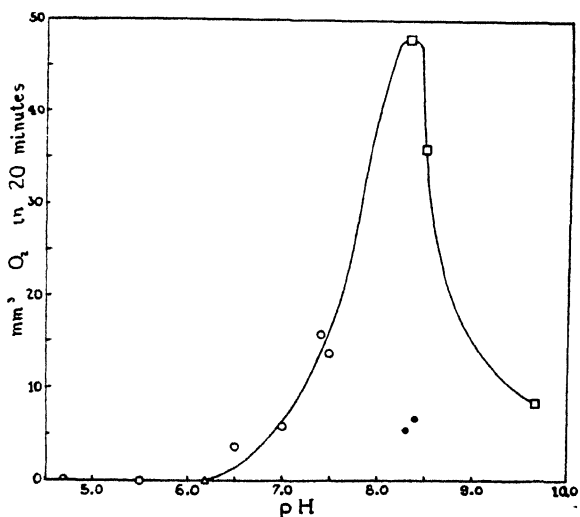


FIG. 3. Rate of oxygen uptake as a function of pH. Each manometer cup contained 0.8 cc. of enzyme, 0.1 cc. of 10 mg. per cent flavin adenine dinucleotide, 0.3 cc. of M glycine, and 2 cc. of 0.5 M buffer. Final volume 3.2 cc. The following symbols indicate the nature of the buffer: ● veronal, ○ phosphate, □ dimethylglycine, △ β -glycerophosphate, and ◐ acetate.

enzyme almost completely. Unfortunately, none of the other buffers employed was reliable in the pH range of 7.5 to 8.5. To minimize the buffering action of the phosphate ions in the stock enzyme preparation, the purification was carried one step further by acidifying to pH 4.6 and treating with sodium sulfate (19 gm. per 100 cc.). The precipitate containing the enzyme was dissolved in water and the solution neutralized. Such an enzyme preparation did not shift the pH values of the 0.5 M buffers with which it was mixed in the proportions of 1:2. The pH values with all components present were measured electrometrically at the end of the manometric runs.

The hydrogen ion concentration may affect not only the rate of oxidation of glycine by the enzyme but among other factors the rate or extent of combination of FAD with the specific protein. In other words, since the enzyme has to be reconstituted before it can function, there is no way of deciding from such an experiment whether the hydrogen ion concentration is affecting the formation of the enzyme, the activity of the enzyme, or both. A pH curve with an unsplit enzyme, however, would make it possible to distinguish between these two effects.

Capryl alcohol (saturated aqueous solution), sodium fluoride (0.2 M), zinc sulfate (0.001 M), iodoacetic acid (0.001 M), cyanide (0.01 M), and sulfadiazine (0.005 M) have no action on the enzyme. 0.01 M iodoacetic acid inhibits 30 per cent. Copper sulfate inhibits 100, 64, and 37 per cent at respective concentrations of 0.001 M, 0.0002 M, and 0.0001 M.

The glycine enzyme tolerates a 3 minute exposure to 55° without any appreciable loss of activity. The same exposure to 60° leads to a loss of 75 per cent of the original activity.

Reversible Splitting—Glycine oxidase prepared from pig kidney by the procedure outlined in the experimental section is inactive unless supplemented with FAD. Other prosthetic groups like adenylic acid, diphosphopyridine nucleotide, diphosphothiamine, and riboflavin cannot replace FAD. The relationship between added FAD and the rate of oxygen uptake is shown in Fig. 4.² From the slope of the linear part of the curve it appears that 8.5 c.mm. of oxygen are taken up in 10 minutes for each microgram of flavin phosphate equivalent of FAD. This is less than one-fourteenth of the value which obtains for the activity of FAD in the reconstructed *d*-amino acid oxidase system (10). A discrepancy of this order of magnitude left open the possibility that some impurity in the FAD preparation was the active principle and not FAD. This possibility, however, was ruled out by the demonstration that the activity per microgram of FAD was identical whether the sample of FAD was prepared from bakers' yeast by the method of Warburg and Christian (11) or from purified

² The fall in velocity at higher concentrations of FAD has been observed consistently. Its significance is obscure.

xanthine oxidase by liberation from the protein with which it was combined (12).

The comparative ease with which the glycine enzyme prepared from pig kidney is split under the relatively mild conditions employed in the purification procedure and the close resemblance of Fig. 4 to a dissociation curve might be regarded as evidence that glycine oxidase is a dissociating flavo-protein at neutral or slightly alkaline pH. However, our experience with the properties of glycine oxidase prepared from sources other than pig lead us to the opposite conclusion; *viz.*, that it does not dissociate appreciably over the pH range of 4 to 9. This conclusion is based on the fact that

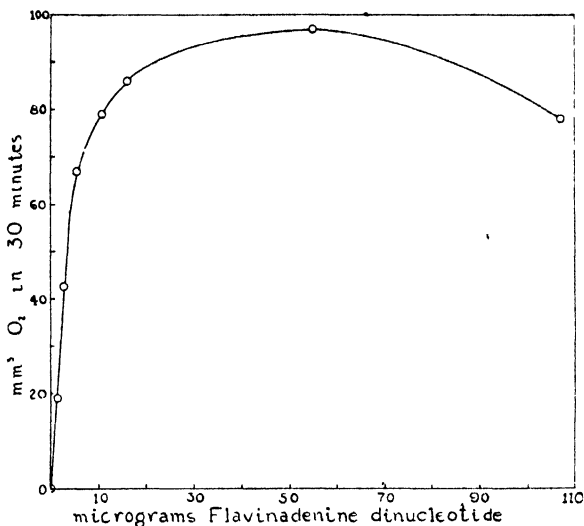


FIG. 4. Rate of oxygen uptake as a function of the concentration of flavin adenine dinucleotide. Each manometer cup contained 1 cc. of enzyme, 0.5 cc. of 0.5 M dimethylglycine buffer, pH 8.3, and 0.5 cc. of M glycine. Final volume 3 cc.; 38°; alkali in center well.

preparations of the enzyme from lamb, human, dog, cat, and ox kidney are not split at all by the identical procedure which completely splits the enzyme from pig kidney. While this discrepancy might argue some unique properties for the pig enzyme, the available evidence is consistent with the assumption that the element of difference is not the oxidase but some factor present in crude preparations of pig kidney which breaks the linkage between protein and the prosthetic group possibly by irreversible destruction of FAD. Indeed by alteration of the method of preparation we have succeeded in preparing glycine oxidase from pig kidney which is not split at all. Thus, whereas ammonium sulfate precipitation of the enzyme from a

neutral extract of the acetone powder leads to an almost completely split enzyme, sodium sulfate precipitation from an acetate (pH 4.8) extract of the acetone powder yields unsplit enzyme. If an equilibrium dissociation of the flavoprotein were involved, precipitation in acid solution should have been more efficacious in splitting the enzyme than precipitation in neutral solution. Since the reverse was true, we must postulate some destructive agent or factor which operates at neutral pH and which is suppressed at pH 4.8. Just as glycine oxidase may be wholly dissociated when prepared from pig kidney but not from other kidneys, so *d*-amino acid oxidase shows the same discrepancy. The comparatively mild conditions needed to split *d*-amino acid oxidase of pig kidney (pH 4 to 7) are inadequate to split the same enzyme from other sources. In fact, pH 2 is still not acid enough to split the *d* enzyme of lamb kidney. Furthermore, partially split glycine or *d*-amino acid oxidase prepared from pig kidney always becomes more completely split on storing. Whatever the nature of this irreversible destruction of bound FAD, it apparently proceeds at a not inconsiderable rate in crude pig kidney preparations. Ease of splitting is undoubtedly a consequence of this destruction of bound FAD. It is of interest that Straub's method (13) for the preparation of split *d*-amino acid oxidase from pig kidney depends not upon strong acid conditions but primarily upon allowing the enzyme to stand around at room temperature long enough for inactivation of bound FAD to take place. Consistent with the above interpretation is the fact that purified unsplit preparations of both the *d* and glycine enzymes from pig kidney are not split by the same procedures which readily split crude preparations from the same source. In other words, when special precautions have been taken to prevent splitting in the early stages of purification, pig kidney preparations are indistinguishable from those of other sources as far as conditions of splitting are concerned.

While both the glycine and *d* enzymes are split in crude extracts of pig kidney, the time factor is different in the two cases. Whereas the glycine enzyme can be largely split in a matter of hours, some days must elapse before the *d* enzyme in the same preparation is completely split.

It can be readily demonstrated that rates of reaction and not equilibria determine the form of Fig. 4 and hence that the resemblance to a dissociation curve is only apparent. FAD attains its maximum catalytic activity in the fully conjugated enzyme. If in a reconstructed system the activity per microgram of FAD in the presence of excess of other components is the same as in the unsplit conjugated enzyme, we may assume that equilibrium conditions obtain in the reconstructed system. If, however, there is a considerable discrepancy, then clearly kinetics, not dissociation equilibria, determine the catalytic activity of varying amounts of FAD in the presence of excess of specific protein. To evaluate the maximum catalytic activity

of FAD in the unsplit glycine enzyme, the preparation must be freed of other flavoproteins. Since, however, the preparations used to obtain this figure still contained considerable amounts of other flavoproteins, our estimates of the activity of FAD in the unsplit enzyme are minimal. Yet even so, the value of FAD activity (expressed as c.mm. of oxygen per 10 minutes per microgram of flavin phosphate equivalent of FAD) was about 4.5 times as great in the enzyme of the intact lamb kidney as in the reconstructed enzyme; *i.e.*, 38 c.mm. of oxygen instead of 8.5. Judging by the relative activities of glycine oxidase and other flavoproteins present in the unsplit lamb enzyme, the difference may be as large as 15-fold if not more. The conclusion is therefore obligatory that kinetics of combination determine the form of the curve in Fig. 4.

Distribution of Enzyme—No systematic survey of the enzyme in animal tissues has been made, but the enzyme already has been prepared from the following sources: cat kidney and liver, dog kidney, lamb kidney, ox kidney, rat liver, pig kidney, human kidney, rabbit kidney and liver. The highest concentration of the enzyme was found in cat kidney. Rat kidney was the only kidney tested which failed to yield an active preparation.

Oxalic Acid Formation from Glyoxylic Acid—Since the existence of glycine oxidase establishes glyoxylic acid as a normal metabolite, we carried out experiments to throw some light on the metabolic fate of glyoxylic acid. We have found two systems which catalyze the rapid oxidation of glyoxylic acid to oxalic acid: (1) xanthine aldehyde oxidase of milk (14) and liver (12), and (2) a mutase of rabbit skeletal muscle which does not appear to be identical with any enzyme yet described. The rate of oxidation of glyoxylic acid by milk aldehyde oxidase is about as rapid as that of acetaldehyde which is the most rapid substrate (*cf.* Table II). The relationships between oxygen consumption, disappearance of glyoxylic acid, and formation of oxalic acid are given in Table III. Rabbit skeletal muscle contains an enzyme which under anaerobic conditions oxidizes glyoxylic acid to oxalic acid (*cf.* Table IV). From the relationship between CO_2 evolution in a bicarbonate medium, disappearance of glyoxylic acid, and formation of oxalic acid, it would appear that a dismutation is taking place. Approximately 1 mole of CO_2 is liberated for each 2 moles of glyoxylic acid disappearing and for each mole of oxalic acid formed. The mutase is not inhibited by iodoacetic acid and hence is not identical with glyoxalase, triose dehydrogenase, or aldehyde mutase (15, 16). To establish in a more convincing way that oxalic acid is formed from glyoxylic acid we carried out some experiments with tissue slices of rat kidney and liver. Very rapid formation of oxalic acid from glyoxylic acid was observed anaerobically in the presence of kidney slices. However, the rate of oxalic acid formation in the presence of liver slices was somewhat slower. In both cases

boiled controls failed to produce oxalic acid. There seems little doubt that formation of oxalic acid can be accounted for by oxidation of glycine via glyoxylic acid. It is of interest that the kidney which is often the

TABLE II

Oxidation of Glyoxylic Acid by Milk Xanthine Aldehyde Oxidase

The experiments were carried out anaerobically in Thunberg tubes at 38°.

	Tube 1	Tube 2	Tube 3	Tube 4
	cc.	cc.	cc.	cc.
Enzyme.....	1.0	1.0	1.0	
Boiled enzyme.....				1.0
0.05 M glyoxylic acid.....			1.0	1.0
0.1 M acetaldehyde.....		1.0		
0.1% methylene blue.....	0.1	0.1	0.1	0.1
Water.....	1.0			
	min.	min.	min.	min.
Decolorization time.....	∞	3.0	3.25	∞

TABLE III

Relationship between Oxygen Uptake, Disappearance of Glyoxylic Acid, and Formation of Oxalic Acid by Aldehyde Oxidase of Milk

The three mixtures were aerated for 4½ hours at 38° and the experiment terminated by addition of trichloroacetic acid. Oxygen uptake was measured manometrically in an aliquot.

	Cup 1	Cup 2	Cup 3
	cc.	cc.	cc.
Milk oxidase.....	20		20
Boiled milk oxidase.....		20	
0.05 M glyoxylic acid.....	10	10	
Red cell suspension*.....	1.0		1.0
Boiled red cell suspension.....		1.0	
Water.....			10
	micromoles	micromoles	micromoles
Oxygen absorbed.....	423	42	14
Glyoxylic acid utilized.....	414	19	
Oxalic acid formed.....	350	7	10

* The red cells provide a convenient and potent source of catalase for protection of the oxidase from H_2O_2 .

site of oxalic acid stones should be rich both in the enzyme which forms glyoxylic acid by oxidation of glycine and in the enzyme which forms oxalic acid by oxidation of glyoxylic acid.

EXPERIMENTAL

Preparation of Glycine Enzyme—Fresh pig kidneys are divested of fat and minced with $\frac{1}{2}$ volume of water in a Waring blender to a fine paste and poured into 5 volumes of acetone cooled to -10° with dry ice. The mixture is rapidly filtered with suction on large Buchner funnels. The cake of tissue is washed several times with small portions of cold acetone, and is then removed from the filter paper and broken up finely until dry. Connective tissue fibers are separated from the powder and discarded. The powder is resuspended in 5 volumes of acetone. After 10 minutes the mixture is again filtered with suction. The fat-free cake is broken up finely until dry.

TABLE IV

Oxidation of Glyoxylic Acid by Mutase of Rabbit Skeletal Muscle

The experiments were carried out for 5 hours at 38° with a gas mixture of 95 per cent N_2 and 5 per cent CO_2 bubbling through the solutions. Formation of carbon dioxide was measured manometrically in an aliquot.

	Cup 1	Cup 2	Cup 3
	cc.	cc.	cc.
Muscle enzyme.....	20		
Boiled muscle enzyme.....			20
M sodium bicarbonate.....	2.5	2.5	2.5
0.05 M glyoxylic acid.....	20	20	20
Water.....		20	
	micromoles	micromoles	micromoles
CO_2 evolved.....	395	11	6
Glyoxylic acid utilized.....	830	63	
Oxalic acid formed.....	462	64	6

250 gm. of acetone powder are mixed thoroughly with 2.5 liters of water. After 30 minutes, the mixture is filtered and the precipitate is washed with small portions of water. Ammonium sulfate (30 gm. per 100 cc.) is added to the filtrate (about 2.5 liters). The precipitate is filtered off and redissolved in 800 cc. of water. Monopotassium acid phosphate (24 gm. per 100 cc.) is added, the precipitate being centrifuged off and resuspended in 300 cc. of water. Sodium carbonate (10 per cent) is added dropwise until the mixture is brought to about pH 8.2. The insoluble material is centrifuged off and discarded. The precipitation procedure with monopotassium acid phosphate is repeated. The final enzyme solution (about 150 cc.) is pale greenish yellow in appearance but water-clear.

While the enzyme can be prepared from other sources, pig kidney has

been found to be the most satisfactory source. The fresh pig kidneys may be stored indefinitely when kept frozen on dry ice. There are three important precautions to be observed in the preparation: (1) the mince prepared in the Waring blender must be very fine but should not be allowed to warm up above 30°; (2) the first acetone precipitation must be carried out below 0° and the cake must not exceed that temperature until dry; (3) the acetone powder must be completely fat-free if the salt precipitations are to go smoothly.

The enzyme preparation retains activity for 7 to 10 days when kept at 0–5°. Dialysis against distilled water rapidly leads to great loss of activity; *e.g.*, 80 per cent loss after 12 hours. Dialysis against 0.05 M phosphate buffer (pH 7.4) is attended with a slower loss of activity; *e.g.*, 40 per cent loss after 12 hours.

Preparation of Other Enzymes—For studying the enzymic oxidation of glyoxylic acid the xanthine oxidase of milk was prepared by the method of Corran *et al.* (12) and the mutase of rabbit skeletal muscle by the method of Green *et al.* (17).

Methods of Estimation—The values for volatile base and keto acids given in Table I were estimated in a trichloroacetic acid filtrate of the enzyme mixture obtained at the end of the manometric runs. Both methylamine and NH_3 were determined by the micro-Kjeldahl procedure and glyoxylic acid by a micro adaptation of the bisulfite-binding method of Clift and Cook (18). According to Long (19) 1 cc. of 0.1 N iodine is the equivalent of 0.625 cc. of 0.1 M glyoxylic acid. This equivalence has been assumed in calculating the results. Though the enzyme blanks gave insignificant values for oxygen uptake and keto acid formation, detectable amounts of NH_3 were present. This varied with the degree of alkalinity and undoubtedly arises from hydrolysis of amide groups in the protein. After 130 minutes at pH 8.3 and 8.8 volatile base amounting to 5.3 and 8.9 micromoles, respectively, was obtained. The values in Table I, observed at pH 8.3, are corrected by the appropriate amount.

In following the enzymic oxidation of glyoxylic acid to oxalic acid, the former was estimated as described above. Oxalic acid was estimated after an aliquot of the trichloroacetic acid filtrate was neutralized with dilute NH_3 . Excess CaCl_2 was added and after the mixture had stood at 0° overnight calcium oxalate was separated and washed by centrifugation. Oxalate was then determined in the usual manner by titration with potassium permanganate.

Isolation of Glyoxylic Acid 2,4-Dinitrophenylhydrazone—The same procedure was followed for the isolation of glyoxylic acid from both glycine and sarcosine runs. A large scale enzyme mixture was set up, containing 80 cc. of enzyme, 16 cc. of M substrate, and 4 cc. of 20 mg. per cent flavin

dinucleotide. The final pH was adjusted to 7.8. The mixture was aerated for 4 to 8 hours at 38° and the progress of the oxidation was followed manometrically in a pilot run. At the end of the experiment, 0.5 volume of 6 N HCl was added, and the clear filtrate was mixed with the theoretical amount of 2,4-dinitrophenylhydrazine dissolved in 2 N HCl. The crude hydrazone (65 per cent yield) was recrystallized from aqueous alcohol; m.p. 187–188° for the sample from the experiment with glycine and 192° for the sample from that with sarcosine. This derivative apparently exists in two isomeric forms, one melting at 202° (20) and the other at 190° (21). The melting points obtained in our samples undoubtedly represent mixtures of the two isomeric forms but both samples were pure, as indicated by the analytical data. Mixed melting points were therefore not feasible.

<i>Analysis</i> — $C_8H_{10}O_6N_4$.	Calculated.	C 37.8, H 2.38, N 22.1
	Found from glycine.	" 38.0, " 2.28, " 21.9
	" " sarcosine.	" 38.0, " 2.44

Isolation of Methylamine Picrolonate—In a large scale run such as was described above sarcosine was aerated for 8 hours at 38°. Care was taken to have the enzyme completely free of traces of NH_3 . A manometric pilot run showed that the oxygen uptake of the mixture corresponded to the formation of 120 mg. of methylamine. Estimation of methylamine in an aliquot disclosed 110 mg. The mixture was deproteinized by addition of 0.5 volume of 6 N H_2SO_4 . The protein-free filtrate was found to contain no NH_3 as evidenced by a negative Nessler's reaction.³ The filtrate was then made alkaline with 40 per cent sodium hydroxide and steam-distilled in a Kjeldahl apparatus for 20 minutes, the tip of the condenser dipping into 20 cc. of alcohol containing an equivalent of picronic acid (936 mg.). The picrolonate solution was concentrated to a small bulk and allowed to crystallize. The yield of crude picrolonate was 650 mg. After three recrystallizations from aqueous alcohol, the melting point of the picrolonate was 241.5°. It showed no depression on admixture with an authentic sample which also melted at 241.5°.

<i>Analysis</i> — $C_{11}H_{11}O_6N_3$.	Calculated.	C 44.7, H 4.44
	Found.	" 44.6, " 4.48
	"	" 44.6, " 4.37

Chemical Preparations—The compounds referred to in the above sections were prepared by the following methods: dimethylglycine by the method

³ Though the enzyme preparation had been rendered NH_3 -free by repeated precipitation with KH_2PO_4 , small amounts of NH_3 formed by hydrolysis of amide groups in protein were fractionally distilled away during the 8 hour incubation period at 38° and pH 8.3.

of Michaelis and Schubert (22), coenzyme I by the method of Williamson and Green (23), muscle adenylic acid by the method of Lindner (24), and FAD by the method of Warburg and Christian (11). We are grateful to Merck and Company, Inc., for a supply of riboflavin and diphosphothiamine. The samples of glycylglycine and leucylglycylglycine were gifts from Dr. N. W. Pirie. An aqueous solution of glyoxylic acid was prepared from oxalic acid by reduction with magnesium powder by the method of Benedict (25). It was then extracted with ether and purified through the calcium salt by the method of Debus (26). The preparation used was an equimolar mixture of glycolic and glyoxylic acids.

SUMMARY

Glycine oxidase is a flavoprotein which catalyzes the oxidation of glycine to glyoxylic acid and NH_3 , and the oxidation of sarcosine to glyoxylic acid and methylamine. Under appropriate conditions, the protein can be separated reversibly from the flavin component, *viz.* flavin adenine dinucleotide. Over the pH range of 4 to 9 glycine oxidase appears not to dissociate to any significant extent. The enzyme has been found in liver or kidney of all animals tested. Systems which catalyze the oxidation of glyoxylic acid to oxalic acid have been studied.

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STUDIES OF THE METABOLISM OF MANNOSE
THE INFLUENCE OF MANNOSE ADMINISTRATION ON BLOOD SUGAR,
BLOOD LACTIC ACID, AND LIVER GLYCOGEN IN
THE ADULT RABBIT

By WILLIAM H. BAILEY, 3RD,* AND JOSEPH H. ROE

(From the Department of Biochemistry, School of Medicine, George Washington
University, Washington)

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Mannose, glucose, and fructose are closely related and have long been known to be interconvertible *in vitro* (1). The metabolism of glucose and fructose in the mammal has been extensively studied, whereas mannose has been the subject of relatively few, partial studies *in vivo*. The finding of mannose in certain compounds of special physiological import, such as egg white proteins (2), skin collagen (3), human, anterior pituitary gonadotropic hormone (4), and phosphatides of tubercle bacilli (5), adds interest to metabolic studies of this sugar. Mannose has been shown to be readily fermentable by yeast (6-11) and readily glycolyzed by various tissues and tissue extracts *in vitro* (12-18). There are indications in the literature which point to the direct utilization of mannose by the mammal. The results of Mann and coworkers (19) and others (20,21) on the prevention of hypoglycemic shock after hepatectomy are in line with this. Certain workers have shown that mannose is a direct physiological antagonist to insulin (22).

Slow absorption of mannose has made tolerance studies difficult in the rat and in man; in addition doses of 50 gm. produce diarrhea in man (23). Harding *et al.* abandoned their work on man on this account (23). In two experiments conducted on the human subject by the present writers (unpublished) confirmation of the diarrheal effect of mannose was found. For these reasons the rabbit was used as an experimental animal in the present work. The absorption of mannose by the rat has been recently studied by Deuel *et al.* (24); the only available study on the rabbit is that by Hédon in 1900 (25). A reinvestigation of the absorption of mannose in the rabbit by the technique of Cori (26) should be carried out.

Harding and coworkers in 1933 conducted studies on mannose tolerance in man, but found no mannemia and no hyperglycemia (23). Deuel *et al.*

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in 1938 (24) studied the ketolytic and glycogenic ability of mannose in rats, but reported no blood sugar data. In view of the incompleteness of data upon the metabolism of mannose it was considered of interest to follow mannose tolerance and glycogenesis along with the determination of some intermediate blood metabolite. In the present paper three lines of investigation are reported; namely, the effects of mannose administration on blood sugar, blood lactate, and liver glycogen. This work was done on rabbits in an attempt to discover how mannose is utilized.

EXPERIMENTAL

Methods and Reagents

Mannose used in the experiments reported in this paper was prepared by the authors. After a number of studies the method adopted was as follows: Pure α -methylmannoside was prepared from vegetable ivory meal¹ according to the method of Hudson (27). Two recrystallizations were found necessary to give a pure product as judged by specific rotation, $[\alpha]_D^{20} = 80.8^\circ$. 500 gm. of this mannoside were then hydrolyzed by refluxing in 10 liters of 20 per cent formic acid for 50 hours. The orange hydrolysate was then concentrated to 2 liters, decolorized with about 20 gm. of acid-washed norit, and concentrated at 10 mm. of Hg and 40° to a thick, colorless syrup. The syrup was taken up in 500 to 600 cc. of glacial acetic acid and crystallized as in the method of Hudson and Jackson (28). About 200 gm., dry weight (43 per cent yield), of pure *d*-mannose were obtained, $[\alpha]_D^{20} = 14.8^\circ$. The low yield (due to incomplete crystallization of the syrup) was of minor importance, since a product was obtained which was prepared without the introduction of toxic substances such as heavy metals, and which was pure without recrystallization. About 800 gm. of mannose were prepared in the above manner.

Blood sugar was determined by the Benedict 1931 method (29), modified slightly for adaptation to the Evelyn colorimeter; *i.e.*, unknowns and standards were adjusted to contain 0.1 to 0.2 mg. of sugar, and a blank of all reagents was run with each experiment. The glucose used for analytical standards was C.P. anhydrous grade. Glucose for metabolism studies was U.S.P. granular. Fructose was determined by the method of Roe (30). Estimations of urinary sugar in the carbohydrate balance studies were carried out by the Benedict quantitative method (31).

Differential glucose and mannose determinations were carried out by the method of Harding, Nicholson, and Armstrong (23) as modified by Nicholson and Archibald (32); a strain of *Proteus vulgaris* from a collection of desiccated bacteria was used, and the concentration of reducing substances

¹ Kindly donated by the Art-in-Buttons Company, Rochester, New York.

was measured by the Benedict method, as described above. It was found necessary to plan animal experiments after the organisms were cultured to a favorable state of activity as determined by glucose "removal" tests.

Lactic acid was determined on tungstomolybdate (Benedict) blood filtrates by the method of Friedemann, Cotonio, and Shaffer as given by Peters and Van Slyke (33), with the apparatus of West (34). Pure zinc *dl*-lactate was prepared and used as a primary standard for recovery tests run with each group of blood lactate estimations. Liver glycogen was determined by the method of Good, Kramer, and Somogyi (35).

Metabolism Studies

The first problem investigated was the effect of mannose ingestion on blood sugar. This question was studied by experiments on carbohydrate balance in which the concentrations of blood glucose and blood mannose were followed over periods of 4 to 10 hours after mannose administration, and in which total urinary excretion was measured for periods of 24 to 36 hours after the start of each experiment. Eight experiments were carried out on five different animals; in five of these experiments differential glucose and mannose determinations were run on each blood sample.

The procedure used in each case is outlined in the following. Animals were fasted 36 to 48 hours and placed in metabolism cages. Control blood samples were taken and mannose was given by stomach tube within 10 minutes in dosages of 2 to 5 gm. per kilo of body weight in 10 per cent solution in water. Blood samples were subsequently taken at 30 or 60 minute intervals for the duration of the experiment. All samples were obtained by bleeding from the marginal ear vein into oxalated bottles, and were placed in the refrigerator at once. In most cases the protein-free filtrates were made the following day. Animals were kept in the metabolism cages, and after the last blood sample water was allowed during the subsequent period of urine collection. For the differential sugar determinations 5 cc. aliquots of the 1:10 tungstomolybdate filtrates were incubated with *Proteus* as in the method of Nicholson and Archibald (32) and 5 cc. aliquots of each blood sample were treated identically except for the addition of the bacteria. The reducing substance in these filtrates was then determined by the Benedict method, on 2 cc. of the bacterially treated and 1 cc. of the untreated filtrate (plus 1 cc. of water) in Folin-Wu sugar tubes. The observed total sugar is the concentration of reducing substance found for the untreated filtrate read against a glucose standard. The residual reducing substance after incubation with *Proteus* read against a mannose standard is the value for mannose plus saccharoid. The saccharoid value (determined for each experiment) is the concentration of residual reducing substance found for the fasting blood sample after treatment with *Proteus*.

Observed total sugar values are corrected by adding a fraction of the mannose concentration in the same sample. The correction is determined by multiplying the mannose value by the per cent difference in reducing power between glucose and mannose, as in the calculations of Roe and Schwartzman (36). Such a correction is valid, since the mannose fraction of the total blood sugar was underestimated by a similar percentage by being read against a glucose standard. In each experiment the actual difference between the reducing values of the glucose and mannose standards is the factor used in calculating the corrected total sugar. Such a procedure is considered more reliable than using average reduction equivalents, since in this method each experiment acts as its own control. The corrected total

TABLE I
Blood Glucose and Blood Mannose after Mannose Ingestion

Animal 5, male rabbit, 2.14 kilos, fasted 48 hours. Dose, 4 gm. of mannose per kilo, in 100 cc. of H₂O.

Time (1)	Blood sugar values, mg. per 100 cc. whole blood					
	Observed total (2)	After <i>Proteus</i> (3)	Correction, (7) $\times$ 0.12* (4)	Corrected total sugar (5)	Blood glucose (6)	Blood mannose (7)
<i>min.</i>						
0	85	14†		85	71	0
60	158	30	1.9	160	130	16
120	138	35	1.6	140	105	21
180	124	29	1.5	126	97	15
255	126	21	0.8	127	106	7

* Copper reduction values: glucose 100, mannose 88.

† Saccharoid value for this experiment.

sugar value minus the "mannose plus saccharoid" value represents blood glucose.

The results of one of the experiments are shown in Table I. Column 4 illustrates the method of calculating the correction explained above. The saccharoid determination serves also as a check upon the activity of the microorganisms. The average saccharoid value found in samples of fasting blood from seven different animals was 10 mg. per cent (range, 6 to 14 mg. per cent). Added mannose was recovered from blood filtrates to the extent of 93 to 107 per cent in tests run on three different batches of bacteria. An assumption made in the differential sugar determinations in these experiments is that the saccharoid value remains the same for the duration of the experimental period. Control experiments on non-glucose reducing substance as determined with *Proteus* showed that it remained very constant over periods of at least 3 hours.

Inspection of Table I shows that the administration of mannose by mouth leads to a prolonged hypersaccharemia (Column 5), an elevation of blood glucose (Column 6), and the appearance of mannose (Column 7) in the peripheral venous blood of an intact rabbit. Four other experiments in this series showed similar results except in the degree of metabolic transformation of mannose. The experiment of Table I showed the greatest increases in blood glucose, but the other four experiments gave significant changes in the same direction. In one of these experiments in which the samplings were continued for 10.5 hours the hyperglycemia and mannemia still persisted.

The total sugar excretion over periods of 24 to 36 hours after mannose administration in doses of 2 to 4 gm. per kilo by the oral and intraperitoneal routes averaged 4 per cent of the dose administered (range, 0 to 8 percent) in eleven animals tested. Differential sugar determinations were not done on these urines because of the small amounts of reducing substance found.

The specific fructose method of Roe (30) was applied to blood samples in one of the experiments on mannose tolerance. It was found that no measurable fructose was present in any blood sample during the 4 hour period of the test. Blood samples from four other animals receiving doses of mannose of 3 to 5 gm. per kilo of body weight also showed blood fructose values of zero.

The second phase of this investigation was a study of the effect of mannose administration on blood lactic acid. Carpenter *et al.* (37) found that glucose, galactose, and fructose give rise to increased lactate in the blood under basal conditions. Rynbergen and coworkers (38) suggested that lactic acid might be an intermediary in the transformation of fructose to glucose. Therefore, it was considered advisable to study the effect of mannose administration on blood lactate in the resting rabbit. Five experiments on the effect of mannose on blood lactate were carried out, along with two positive controls on the effect of glucose, and one negative control on the effect of mannoheptose. Total blood sugar and blood lactic acid were determined on all samples.

The experimental procedure was as follows: Rabbits weighing about 3 kilos each were fasted for 24 hours. The animals were maintained in a state of mild sedation by means of small doses of veterinary nembutal solution given intraperitoneally every hour or so, depending on the condition of the animal. For the duration of the experiment the animal was in a relaxed, reclining position, the respiration was regular, the pupil responded to bright light, and pain reflexes were never absent. It was felt that such a state was the closest approach to a basal condition that could be obtained. 2 or 3 hours of control period were allowed before the sugar was given to permit the animal to come to a steady state as far as blood sugar

and blood lactate were concerned. Analysis of blood samples taken hourly during the control period showed that the values for blood sugar and blood lactate remained constant or dropped slightly; thus increases after sugar administration could be regarded as true increases above the basal level. Sugars were given in dosages of 2 gm. per kilo of rabbit, and 5 cc. blood samples were taken at hourly intervals over periods of 3 to 6 hours in oxalated bottles containing 50 to 100 mg. of sodium fluoride. The fluoride-preserved bloods were placed at once in the refrigerator and analyzed the following day. The α -*D*-mannoheptose used in Experiment H was prepared in Dr. C. S. Hudson's laboratory and had a specific rotation of $+68.8^\circ$ with sodium light at 20° . Recoveries of pure zinc lactate were run along with six of the eight experiments and 95, 95, 92, 90, 90, and 91 per cent, respectively, of 0.605 mg. samples of lactic acid were recovered by analysis. All lactate values reported are averages of two determinations on the same blood filtrate.

The results of eight experiments are summarized in Fig. 1. Inspection of Fig. 1 reveals that, in general, elevated levels of blood lactate parallel hyperglycemia when a hexose was used. In the case of mannoheptose, Experiment H, no increase in blood lactate was observed even when a saccharemia of 200 mg. per cent was reached. It should be noted that Experiment H not only represented a control on the effect of hypersaccharemia upon the blood lactate level, but it also served as a control on every phase of the experimental technique. The curve obtained for blood lactic acid in this case is practically a straight line at the basal level. Thus, changes observed in blood lactic acid can be considered as due only to the influence of the sugar given and to the level of saccharemia reached. When mannose was administered intraperitoneally (Experiments C and D), marked increases in blood sugar were observed and correspondingly marked increases in blood lactate were found. In Experiments E, F, and G, when mannose was given orally, less hyperglycemia and smaller increases in blood lactate were observed. It appears that the differences in uptake of the mannose into the blood will account for the differences in blood sugar and lactate in these two sets of experiments. Mannose administration always led to a positive increase in blood lactic acid.

The third phase investigated was the effect of mannose administration on liver glycogen in fasted rabbits. This question was studied by determining the changes in liver glycogen produced in periods of 6, 10, and 12 hours after mannose was given in varying dosages by three different routes.

The procedure carried out is outlined in the following. The double laparotomy technique of Cohn and Roe (39) was used on ten of the animals of this series. By this method a control value for fasting liver glycogen was determined on the same animal in which glycogen deposited after

mannose or glucose administration was measured. Rabbits weighing from 1.7 to 3 kilos, unselected as to sex, were fasted 24 hours, anesthetized with nembutal, and operated on. After a 1 gm. sample was taken from the right liver lobe, the wound was closed, the sugar was given, and the animal was kept in a metabolism cage for the duration of the experiment. After a period of 6, 10, or 12 hours the animal was anesthetized, reopened, and sam-

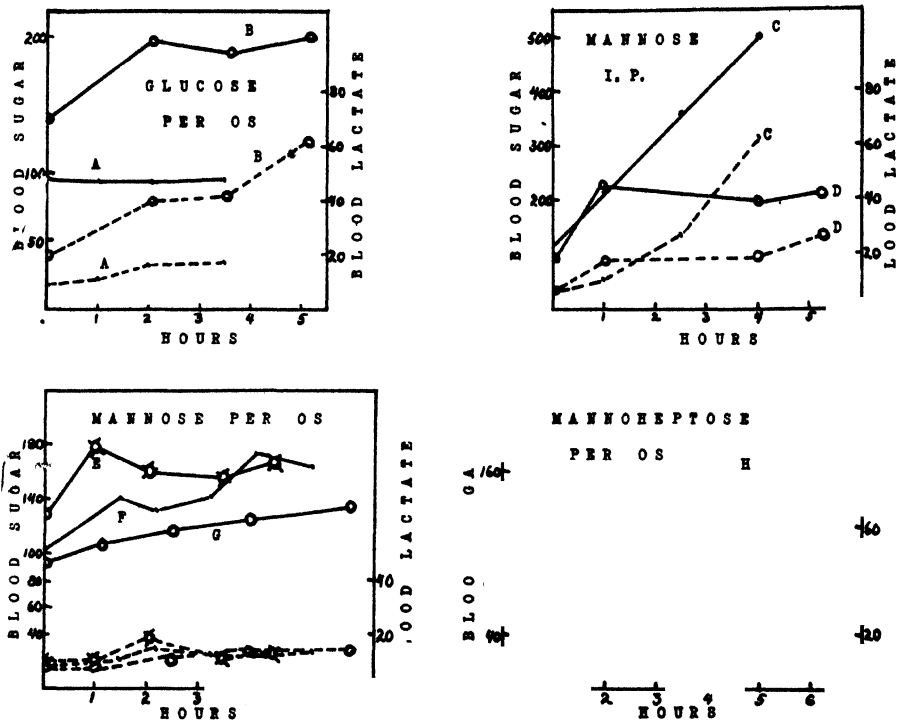


FIG. 1. Influence of administration of various sugars on blood sugar and blood lactate. The doses given in every case were 2 gm. of sugar per kilo of body weight. Each experiment is labeled by letter and represented by a pair of curves. The solid lines refer to blood sugar; broken lines to blood lactate. The units of the vertical axes are mg. per cent.

ples of the right and left liver lobes were taken for analysis. The animal was then sacrificed. All samples were immediately placed in tared tubes containing 5 cc. of 30 per cent KOH, weighed, and subsequently treated as in the method of Good, Kramer, and Somogyi (35). Total sugar excretion was measured on the urines collected during the period of the experiment.

The results of nineteen unselected, serially run experiments are summarized in Table II (Animals 9 and 10 of this series died under anesthesia).

Sugar excretion, when mannose was given by mouth, averaged 4.6 per cent of the dose administered, and 12.2 per cent when mannose was given subcutaneously. The glucose tests serve as positive controls on each of the

TABLE II

Liver Glycogen Formation in Adult Rabbits after Mannose and Glucose Administration by Various Routes

The sugars were given in 20 per cent solution in water; dosages are expressed in gm. per kilo of body weight. The animals are numbered serially.

Sugar	Animal No.	Dose	Liver glycogen, per cent					
			Control	After 6 hrs.	After 10 hrs.	After 12 hrs.	Average	
							Control	After sugar
Sugars given <i>per os</i>								
Mannose.....	5	3	1.05			0.81		
"	6	3	1.11			1.69		
"	7	3	0.61			1.31		
"	8	3	0.49			1.28	0.81	1.27
Glucose.....	11	3	0.79			1.00		
"	12	3	0.32			3.73	0.56	2.37
Mannose.....	13	4			1.40			
"	14	5			2.53		1.97	1.20*
Glucose.....	18	5			4.03			
"	19	4			4.82		4.41	3.64*
Sugars given subcutaneously								
Mannose.....	1	3	0.39	2.99				
"	2	3	1.36	2.93				
"	3	3	1.02			3.81		
"	4	3	0.55			2.90	0.83	3.16
Glucose.....	20	3				1.99		
"	21	3		2.60				2.29
Sugars given intraperitoneally								
Mannose.....	15	3			2.29			
"	16	5			4.11		3.20	2.43*
Glucose.....	17	5			4.15		4.15	3.38

* Based upon the average control value for ten rabbits (0.77).

mannose experiments as shown in Table II. Control values for liver glycogen during fasting averaged 0.77 per cent in ten animals. Negative controls on the effects of length of fast, anesthetic, and operation on liver glycogen carried out by Cohn and Roe (39) in this laboratory showed

that at the 12 hour period *total* liver glycogen values by this technique must be greater than 1.08 per cent to be significant (average of nine experiments). Translating this figure into terms of glycogen *increase* due to sugar administration gives a value of 0.31 per cent ($1.08 - 0.77$) which would be the minimum that could be accepted as significant in these experiments. The last column of Table II, labeled "Average increase," represents glycogen deposited owing to the influence of the sugar administered, and is found by subtracting the value for fasting liver glycogen from the total value found 6, 10, or 12 hours after sugar administration. All values reported for glycogen percentages after sugar administration are averages for samples from two lobes of the liver.

Inspection of Table II shows that mannose given orally at 3 gm. per kilo produced an amount of extra glycogen that is very little greater than the minimum significant value (0.31) under these experimental conditions, but that when the dosage was raised to 4 or 5 gm. per kilo an average of 1.2 per cent of glycogen was deposited during 10 hours. When mannose was given subcutaneously at 3 gm. per kilo it is seen that the glycogen deposited after periods of 6 or 12 hours amounted to 2.33 per cent on the average. The latter figure was higher than that for glucose run under the same conditions. In the last section of Table II it will be seen that the figure for glycogen deposited after mannose administration by the intraperitoneal route compared favorably with that for the glucose experiment. Table II shows in general that in the animals tested mannose raised liver glycogen slightly when given orally, and, when administered parenterally, this sugar was glycogenic to approximately the same degree as glucose.

DISCUSSION

Studies on carbohydrate balance showed that mannose is utilized to a high degree by rabbits, since an average of 96 per cent of varying dosages of the sugar was retained by these animals after administration by the oral or intraperitoneal route. The present studies have shown that mannose was absorbed from the gut and appeared in peripheral venous blood of the rabbit after mannose ingestion. In addition mannose administration produced an elevation of blood glucose. Increases in blood glucose could be interpreted as being due to conversion of mannose to glucose or to stimulation of a glucose-producing mechanism. Stimulation of the formation of blood glucose by a saccharemia of a non-utilizable sugar is unlikely. The data of Roe and Hudson (40) in which a hypersaccharemia was produced by mannoheptose administration showed no significant increase in fermentable reducing substance in the same bloods. Therefore, it is concluded that the data of the present studies can be interpreted as showing conversion of mannose to glucose.

Some insight into a possible mechanism for the metabolic transformation of mannose to glucose is gained from the studies on lactic acid and glycogenesis. Since, by the use of a specific method, no fructose was found in any blood sample from animals tested during the course of hyperglycemia following mannose administration, direct conversion of mannose to glucose by epimerization and attendant Lobry de Bruyn equilibrium appears to be excluded. Rynbergen and coworkers (38) suggested that lactic acid may be an intermediate in the transformation of fructose to glucose. Cori (41) stated that lactate surpasses even fructose in the formation of liver glycogen. The well known lactic acid cycle establishes an important link between the carbohydrate metabolism of muscle and liver. Muscle (and other cellular) glycolysis produces lactate which forms liver glycogen; when liver glycogen is hydrolyzed, glucose results. A plethora of a sugar such as mannose could thus reappear in the blood as glucose after glycolysis in blood and tissues and subsequent glycogenesis and glycogenolysis in the liver. Such a scheme would fit the case of mannose, since blood lactate, liver glycogen, and blood glucose were found to increase after mannose administration.

SUMMARY

1. Studies of the metabolism of mannose in the rabbit have been carried out.

2. Experiments on carbohydrate balance showed an average retention of 96 per cent of doses of mannose of 2 to 5 gm. per kilo when this sugar was administered orally or intraperitoneally. These results indicate a high degree of utilization of mannose by the rabbit.

3. Mannose appeared in the peripheral venous blood and produced an elevation of blood glucose in all animals tested after mannose ingestion. No fructose was found in any blood sample from animals tested during the course of hyperglycemia following mannose administration.

4. Mannose administration led to an increase in blood lactic acid. Elevations in blood lactate produced by mannose were similar to those after glucose administration in that they paralleled increases in total blood sugar. These results were in contrast to those of a control experiment with mannoheptose in which blood lactate remained constant at the basal level even when a saccharemia of 200 mg. per cent was reached.

5. Amounts of liver glycogen found at 6, 10, and 12 hours after mannose administration were approximately of the same order as those found after glucose administration at the same dosage level when these sugars were given parenterally to animals previously fasted 24 hours.

6. The combined results of the experiments carried out indicate that

mannose is fairly slowly absorbed from the gut, well utilized, and convertible to glucose in the intact rabbit. A possible mechanism for the metabolic conversion of mannose to glucose by way of lactic acid and liver glycogen is suggested.

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STUDIES ON THE RELATION BETWEEN VITAMIN M, XANTHOPTERIN, AND FOLIC ACID*

By JOHN R. TOTTER, CARROLL F. SHUKERS, JACK KOLSON,
VIRGINIA MIMS, AND PAUL L. DAY

(From the Department of Physiological Chemistry, School of Medicine, University of
Arkansas, Little Rock)

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A series of reports from this laboratory (2-5) and an independent and simultaneous series of investigations in British laboratories (6-8) have established that the rhesus monkey (*Macaca mulatta*) requires a water-soluble substance (or substances) to prevent leucopenia, anemia, diarrhea, lesions of the mouth and colon, and eventual death. This factor, for which the term vitamin M was suggested, was shown to be distinct from thiamine, riboflavin, and nicotinic acid (3, 4). These observations have been amply confirmed by other workers (9-16). Wilson *et al.* (12) demonstrated that additional supplementation with calcium pantothenate, pyridoxine hydrochloride, choline chloride, pimelic acid, glutamine, inositol, and sodium *p*-aminobenzoate failed to prevent the nutritional cytopenia. Those workers also observed that the intramuscular injection of a "folic acid" concentrate restored normal white cell equilibrium. "Folic acid" concentrates have likewise proved effective in counteracting inhibition of growth in rats caused by feeding succinylsulfathiazole (17). Totter and Day (18) reported that synthetic xanthopterin will partially, but not fully, counteract the succinylsulfathiazole effect and suggested that "folic acid" concentrates might contain xanthopterin. The presence of the pterin in some such concentrates has been confirmed by Mitchell (19).

In a preliminary publication (1) we indicated that xanthopterin has proved at least partially effective in restoring hemocytopoietic function in the vitamin M-deficient monkey, especially when the experimental animal also received a crude source of other factors.

In this communication we wish to present our observations on the rôle of xanthopterin in the nutrition of *Macaca mulatta* and at the same time to present analytical data indicating a dissimilarity in distribution of vitamin M and folic acid. We have also been able to confirm the observa-

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tions of Wilson *et al.* that pantothenic acid, choline, *p*-aminobenzoic acid, pyridoxine, and inositol fail to prevent nutritional cytopenia when they are added to the vitamin M-deficient diet of monkeys.

Methods

Young, immature rhesus monkeys weighing approximately 2 kilos were used for the experiments. The method of care, handling, and selection has been described elsewhere (4). Blood for study was drawn from the animals at weekly intervals or oftener if it was deemed desirable, since we have found the blood picture to be the only reliable criterion of this deficiency. Hematological examinations were made by the same methods as were used by Shukers *et al.* (20). Weight records were also kept, but they are not reported here, as they do not greatly aid in the interpretation of results.

The basal ration (Diet 600) is similar to that used previously and had the following composition (air-dry weight): vitamin-free casein¹ 10 gm., polished uncoated rice 50 gm., ground whole wheat 15 gm., salt mixture (21) 3 gm., sodium chloride 2 gm., and cod liver oil 3 gm. The amount indicated above (83 gm.) was given daily to each animal after being mixed with water and cooked for 2 hours. The method of preparation of the diet has been detailed in an earlier publication (4).

Vitamin supplements to the above diet were given separately in the form of tablets, which the monkeys consumed avidly, or as aliquots of a solution mixed with the diet just before feeding. Except as noted in Table I all the monkeys on which data are here reported received the following: 10 or 20 mg. of ascorbic acid and 1 mg. of thiamine chloride daily and 50 mg. of nicotinic acid and 2 mg. of riboflavin three times weekly. In addition two animals received daily the following (which is indicated in Table II as "vitamin mixture"): 100 mg. of inositol, 50 mg. of choline hydrochloride, 25 mg. of *p*-aminobenzoic acid, 1 mg. of pyridoxine hydrochloride, and 10 mg. of calcium pantothenate. This vitamin mixture was added to the diet just before feeding as an aliquot of 25 per cent alcohol solution of the materials named.

Two monkeys in this series received a daily supplement of 3 gm. of liver powder which was prepared by heating fresh beef liver in an electric oven for 24 hours at 100° and then grinding to a powder. Certain animals received supplements of fresh beef liver, fresh lean beef, or cured ham (Table I). These meats were obtained at local markets weekly and were stored in the refrigerator until used. As needed, they were ground in a meat chopper and mixed with the diet before cooking.

¹ Labco brand, purchased from The Borden Company, 350 Madison Avenue, New York, N. Y.

Xanthopterin was prepared by the method of Purrmann (22) and by a minor modification in which 2,4,5-triamino-6-hydroxypyrimidine bisulfite was used instead of the free base. The bisulfite derivative was obtained by heating the 2,4-diamino-5-isonitroso-6-hydroxypyrimidine (23) from 33 gm. of 2,4-diamino-6-hydroxypyrimidine sulfate (23) with 90 to 100 gm. of sodium hydrosulfite in 100 ml. of water. After 30 to 60 minutes on the steam bath the mixture was diluted, boiled, treated with charcoal, and rapidly filtered while hot. The bisulfite of the triamino base separated rapidly on cooling. 6 gm. of this compound were treated with 10 ml. of water-free dichloroacetic acid and the remainder of the synthesis carried out as described by Purrmann (22).

"Folic acid" assays were made on the basal monkey diet and on the various dietary supplements by the method of Mitchell and Snell (24), taka-diastrase being used to liberate the stimulating factor (25).

Results

Comparison of "Folic Acid" Content of Certain Materials with Their "Vitamin M" Activity—Table I briefly presents data on the folic acid content of the basal vitamin M-deficient diet and of several supplements which have been fed to monkeys, together with statements as to the effectiveness of these supplements in preventing vitamin M deficiency. As measured by the growth of *Streptococcus lactis* R, the daily allowance of basal vitamin M-deficient diet for a monkey (83 gm.) contains 31 γ of folic acid; 5 gm. of dried brewers' yeast, which when fed daily have been shown to prevent nutritional cytopenia in the monkey, contain 21 γ of folic acid. The monkey which received 5 gm. of dried yeast therefore received 52 γ of folic acid daily. On the other hand, the monkeys which received 3 gm. of dried liver powder daily received a total of 170 γ of folic acid daily and yet were not fully protected against vitamin M deficiency. Also, the monkey which received 25 gm. of fresh banana daily was getting 55 γ of folic acid and yet developed nutritional cytopenia in the usual length of time. An examination of Table I reveals other situations in which the folic acid and vitamin M values do not appear to parallel each other.

From these experiments it appears evident that assays for "folic acid," by the method with *Streptococcus lactis* R, do not measure vitamin M activity. This of course does not preclude the possibility that folic acid may be one of two or more substances with vitamin M activity.

The recent isolation of crystalline vitamin B₁₂ by Piffner *et al.* (26) and a substance stimulating growth of *Streptococcus lactis* R by Keresztesy *et al.* (27) does not entirely clarify the status of "folic acid." Since vitamin B₁₂ is an antianemia factor for the chick (26), it is not unlikely that it may

likewise stimulate hemopoiesis in the monkey. However, as this substance is a stimulating factor for *Streptococcus lactis* R (28), it is very probably not always the limiting factor for the monkey in diets producing vitamin M deficiency.

Effectiveness of Synthetic Xanthopterin in Curing and Preventing Nutritional Cytopenia in the Monkey—Table II and Fig. 1 present our data on the effect of xanthopterin on the blood picture of monkeys rendered cytopenic by deficient diets. Monkeys 55 and 129 received xanthopterin in

TABLE I

"Folic Acid" Intake of Monkeys (As Contained in Basal Diet Plus Supplement) Compared with Effectiveness of Diet Supplements in Preventing "Vitamin M" Deficiency

Diet 600 fed throughout.*

Daily supplement	No. of animals	Daily "folic acid" intake, micrograms of material of potency 40,000	Effectiveness of supplement against "vitamin M" deficiency
None†	7	31	Monkeys not protected
" *†	2	50	" " "
5 gm. fresh beef liver†	1	65-101	Monkey fully protected
3 " dried " " †	2	170‡	Monkeys not fully protected
2.5 gm. " brewers' yeast§	1	41	Monkey " " "
5 gm. " " " §	1	52	" fully protected
10 " " " " §	2	73	Monkeys " "
2 " Lilly's liver extract†	1	115	Monkey " "
20 " fresh beef†	1	50	" not protected
10 " cured ham†	1	35	" " "
25 " fresh banana†	1	55	" " "

* In the second group the vitamin-free casein of Diet 600 was replaced by a crude casein (casein, edible, muriatic, manufactured by the Casein Company of America, New York).

† In addition to thiamine, riboflavin, nicotinic acid, and ascorbic acid.

‡ A single large batch prepared from one beef liver.

§ In addition to 4 gm. of orange.

the rapidly fatal, advanced stage of the deficiency. Nevertheless, both showed reticulocyte response and marked increase in both white and red cell counts, but the therapy was unable to prolong appreciably either the restored counts or the lives of these animals.

Monkey 132, after developing the characteristic anemia and leucopenia on the deficient diet supplemented with 25 gm. of banana daily, was given 5 mg. of xanthopterin per day for the remainder of the experiment. The white and red cell counts were raised to nearly normal levels and remained

TABLE II
*Hematological Data on Monkeys Which Received Diet 600
 Supplemented in Various Ways*

Counts were made weekly or oftener but only the most significant data have been included in this table.

Monkey No.	Days on Diet 600	Daily supplement*	White blood cells	Red blood cells	Reticu- lyocytes	Hemo- globin
			thousands per c.mm.	millions per c.mm.	per 1000 r.b.c.	gm. per 100 ml.
55	0	10 gm. fresh beef	6.6	5.38	1	9.23
	15	20 " " "	13.7	5.27	4	11.29
	100		8.4	4.72	3	9.71
	165		8.5	4.46	3	7.80
	170	5 mg. xanthopterin added	6.3	2.92	2	5.14
	174	Xanthopterin increased to 10 mg.	7.5	3.61	12	8.72
	176		10.1	3.94	15	5.78
	178		12.9	2.99	6	6.31
	181		1.9	2.50	2	6.28
	Dead					
129	0	Vitamin mixture†	18.9	4.91		11.30
	21		14.1	4.96	2	7.89
	70		6.3	4.46	1	10.36
	146		19.8	4.55	1	11.10
	203		3.2	3.44	1	9.23
	212	2.5 mg. xanthopterin added	3.7	2.74	2	7.61
	217		4.7	3.70	7	
	218	5 mg. xanthopterin intra- muscularly				
	219		9.4	3.49	30	6.57
	229		1.1	1.75	1	5.05
132	Dead					
	2	25 gm. fresh banana	10.7	4.66	2	8.05
	44		13.2	4.88	2	14.38
	72		6.1	3.01	3	9.02
	84		4.4	3.65	2	7.91
	91	5 mg. xanthopterin added	3.6	3.38	1	8.24
	97		9.5	4.73	45	9.64
	105	Banana withdrawn	10.5	3.87	2	9.09
	121		8.4	4.17	2	9.09
	147		3.6	3.73	1	8.15
	149	1 mg. pyridoxine added	2.3	3.80	3	7.22
	155		4.0	3.78	0	8.10
	167		3.6	3.10	1	6.82
	190		3.6	2.59	10	8.42
	192					
	Dead					

TABLE II—*Concluded*

Monkey No.	Days on Diet 600	Daily supplement*	White blood cells	Red blood cells	Reticu- locytes	Hemo- globin
			thousands per c.mm.	millions per c.mm.	per 1000 r.b.c.	gm. per 100 ml.
137	0	Vitamin mixture† + 1 mg. of xanthopterin	11.2	4.17	1	9.71
	56		7.8	3.77	2	
	104		9.4	4.89	5	8.59
	151		8.9	3.43	4	10.40
	201		11.2	4.13	5	10.65
	249		11.7	3.97	7	10.00
	269		9.8	4.22	4	11.00
	289		5.2	3.20	2	8.80
	Still alive					

* All the monkeys received 20 mg. of ascorbic acid and 1 mg. of thiamine chloride daily, and 50 mg. of nicotinic acid and 2 mg. of riboflavin three times weekly in addition to the supplements shown in this column.

† The daily dose of this vitamin mixture supplied the following: 100 mg. of inositol, 50 mg. of choline hydrochloride, 25 mg. of *p*-aminobenzoic acid, 1 mg. of pyridoxine hydrochloride, and 10 mg. of calcium pantothenate.

there with some fluctuation for almost 30 days. Thereafter the white cell, erythrocyte, and hemoglobin levels steadily declined until the death of the animal on the 192nd day of experiment. The addition of pyridoxine to the diet did not appear to influence the blood picture.

The results obtained with Monkey 128 (Fig. 1) also show, very strikingly, the effect of xanthopterin feeding on the blood counts of the deficient monkey. The effect of heating and drying on the vitamin M content of liver was tested by use of two animals, Monkeys 125 and 128, which received 3 gm. daily of a powder prepared by drying fresh beef liver at 100° for 24 hours and then grinding it to a powder. Monkey 123 served as a control and received fresh liver; for a period of 463 days it received 10 gm. of fresh beef liver daily; thereafter until the present time (205 days) it has received 5 gm. of fresh liver daily, and is still in excellent health, with a normal blood picture.

Monkey 128 developed moderate anemia and marked leucopenia after 220 days on the experimental diet supplemented with 3 gm. of the heated dried liver (Fig. 1). Treatment with 2.5 mg. daily of xanthopterin was followed by a slight reticulocyte response and a steady increase in white and red cell counts, which reached normal levels in about 20 days and remained within normal limits until xanthopterin was withdrawn on the 290th day. Thereafter the counts declined and treatment was resumed on the 324th day. The response to 10 mg. of xanthopterin daily for 3

days was very rapid but somewhat less satisfactory; 2.5 mg. daily for the next 3 months served to keep the counts fluctuating somewhat below previous levels. The second withdrawal was followed by a rather prompt return of moderate anemia and severe leucopenia. Resumption of treatment with 5 mg. of xanthopterin was followed by a marked increase in reticulocytes, and slight increases of red and white cells for a few days. The animal became ill very suddenly on the 462nd day and died 2 days later with a severe leucopenia and the usual signs of vitamin M deficiency at autopsy.

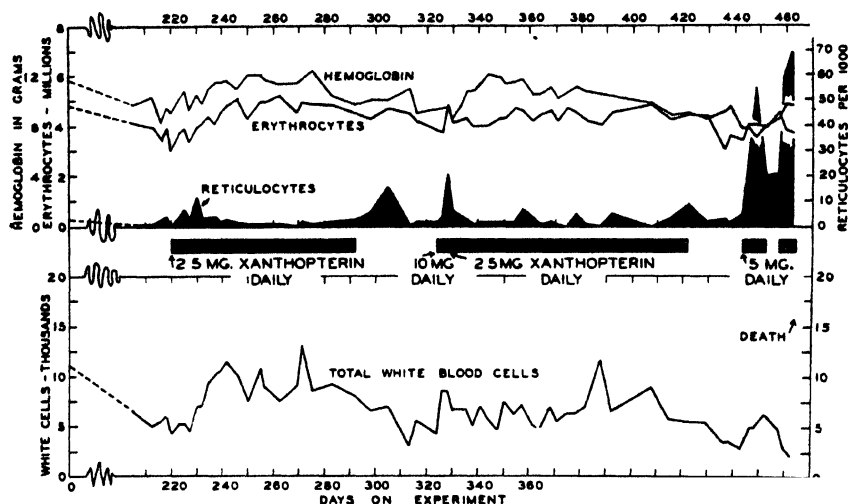


FIG. 1. Hematological data on Monkey 128, male, which received Diet 600 supplemented with 50 mg. of nicotinic acid and 2 mg. of riboflavin three times weekly, and with 20 mg. of ascorbic acid, 1 mg. of thiamine chloride, and 3 gm. of liver powder daily (the liver powder was prepared by drying fresh beef liver at 100° for 24 hours and grinding to a powder). The monkey was given 2.5 mg., 5 mg., or 10 mg. of synthetic xanthopterin daily at the times indicated on the chart.

Monkey 137 (Table II) received 1 mg. of xanthopterin daily from the start of the experiment, in addition to the amounts of ascorbic acid, thiamine chloride, nicotinic acid, riboflavin, inositol, choline hydrochloride, *p*-aminobenzoic acid, pyridoxine hydrochloride, and calcium pantothenate stated in the foot-notes to Table II. The blood counts remained within the lower range of normal for 269 days, but thereafter the blood picture slowly deteriorated. The monkey is still alive on the 289th day but is moderately cytopenic. Although xanthopterin has not completely protected this monkey, it has appeared to delay definitely the onset of nutritional cytopenia.

These data strongly suggest that xanthopterin is required by the monkey for normal hemocytopoiesis. It seems probable that heating and drying liver destroy some other factor or factors likewise required for blood cell formation, in addition to all or part of the xanthopterin known to be present in liver (29).

The increase in leucocytes after injection of a "folic acid" concentrate noted by Wilson *et al.* (12) may have been due wholly or in part to the xanthopterin known to be present in such concentrates; however, our results by no means exclude the possibility that other substances in the concentrate were also active.

SUMMARY

In a number of materials assayed, the distribution of the factor stimulating growth of *Streptococcus lactis* R (folic acid) was shown to be different from that of vitamin M.

Pantothenic acid, choline, *p*-aminobenzoic acid, pyridoxine, and inositol did not prevent nutritional cytopenia in the monkey.

The treatment of cytopenic monkeys with synthetic xanthopterin was followed by reticulocyte responses and increases in red and white blood cell counts.

The results suggest that xanthopterin or some closely allied substance may be required by the monkey for normal hemocytopoiesis; however, it seems probable that unidentified substances may also be necessary.

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THE USE OF STREPTOCOCCUS LACTIS R FOR THE MEASUREMENT OF "FOLIC ACID"*

By T. D. LUCKEY, G. M. BRIGGS, JR., AND C. A. ELVEHJEM
(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

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During studies on two chemically unidentified, water-soluble vitamins necessary for the chick (designated as vitamin B₁₀ and B₁₁ (1)) "folic acid" was determined routinely by the method of Mitchell and Snell (3). Although the method proved satisfactory for assaying similar preparations from liver, the medium was found to be inadequate in several essential factors when other materials were assayed. Since this rendered the assay unreliable under certain conditions, studies were made to see whether the deficiencies of the medium could be eliminated. This paper covers the vitamin, mineral, and other nutrient requirements of *Streptococcus lactis* R² and describes a medium which gives a more reliable assay for "folic acid."

Assay Procedure

The inoculum is prepared directly from a stab culture in a sterile inoculum medium (basal medium plus 200 γ of solubilized liver per 10 ml.) and incubated for 8 to 30 hours at 30°.

A series of duplicate tubes containing 0 to 300 γ of solubilized liver is used to establish a standard curve. Varying amounts of the material to be assayed are selected which are estimated to give a response within the range of the standard curve.

Natural materials often require liberation of the bound neutralite, which may be accomplished by digestion with taka-diaxase, as recommended by the Texas workers (4). 1 gm. of finely ground sample is placed in 8 ml. of 1 per cent sodium acetate solution at pH 4.5 to 4.7. 20 mg. of taka-

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¹ "Folic acid" is the name applied by Mitchell *et al.* to the material found in the norit eluate fraction of spinach which gives growth responses to both *Streptococcus lactis* R and *Lactobacillus casei* on a casein hydrolysate-synthetic medium (2).

² *Streptococcus lactis* Roger 1A at the University of Wisconsin, or No. 8043 of the American Type Culture Collection, Washington, D. C.

diastase in 1 ml. of water are added and the mixture is incubated under toluene for 24 hours at 37°. The digest is then neutralized, autoclaved for 15 minutes at 15 pounds, and diluted as desired.

The samples are placed in tubes $\frac{3}{4}$ by 6 inches, the volume in each is made up to 5 ml., 5 ml. of basal medium are added, and the tubes are sterilized for 15 minutes at 15 pounds pressure. After cooling to room temperature, 1 drop of the inoculum is added to each tube from a sterile pipette, and the tubes are incubated at 30° for 16 hours. Instead of plugging the tubes, it is convenient to put a cover over each rack during sterilization and incubation. Possible contamination during inoculation is of little consequence when this fast growing organism is used in a short period assay.

Response to "folic acid" is measured by use of a photoelectric turbidimeter (*e.g.* an Evelyn colorimeter with Filter 620 (5)). The amount of "folic acid" in the unknown is calculated from the standard curve in terms

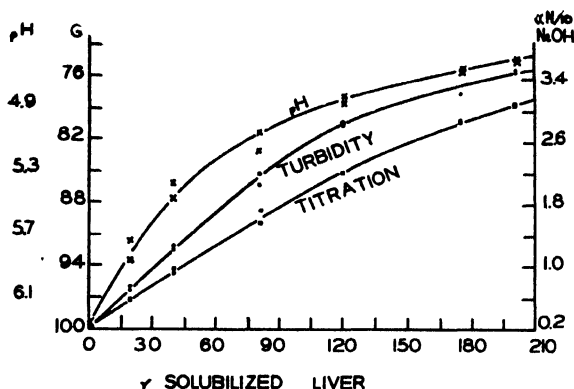


FIG. 1. Comparison of curves obtained by reading turbidity and pH, and titrating one set of tubes. *G* = galvanometer reading (see Table I).

of equivalent amounts of solubilized liver. Three to five concordant results are averaged to give a final value.

We have used two methods of expressing "folic acid" activity. One method gives the values in terms of per cent activity and is based upon the standard, solubilized liver, having 100 per cent activity. The other method involves an empirical procedure used by the Texas group (3) in which the standard liver extract is given a potency of 1 and "folic acid" is assumed to have a potency of 40,000. Thus the activity of the standard is 100 per cent in the first method and in the second method it is 25 γ of "folic acid" per gm.

When a precipitate or a color is formed in the tubes (*e.g.* 10 γ of a copper or a selenium salt will form obstructive precipitates), making turbidity readings inaccurate, the amount of "folic acid" in the sample may be calcu-

lated from either a titration curve or a pH curve. In Fig. 1 the turbidity curve is compared with curves obtained by reading the pH and titrating the same set of standard curves.

Effect of Purines and Pyrimidines—Snell and Mitchell found that *Streptococcus lactis* R required adenine or guanine and thymine for growth (6). Thymine was also shown to be active by Stokstad (7), who found that thymine with adenine, guanine, hypoxanthine, or xanthine partially replaced the *Lactobacillus casei* factor of Snell and Peterson (eluate factor).

TABLE I

Effect of Purines, Pyrimidines, and "Folic Acid" on the Growth of Streptococcus lactis R

Material added	Amount	No. of experiments	G*
None.....		4	98
Adenine.....	1 mg.	4	96
Guanine.....	1 "	6	97
Xanthine.....	1 "	9	97
Uracil.....	1 "	2	96
Thymine.....	2 γ	5	98
Solubilized liver†.....	40 "	4	96
" " (40 γ) + adenine.....	1 mg.	4	92
" " (40 ") + guanine.....	1 "	4	91
" " (40 ") + xanthine.....	1 "	4	91
" " (40 ") + uracil.....	1 "	2	96
" " (40 ") + thymine.....	2 γ	4	97
Thymine (2 γ) + adenine.....	1 mg.	5	85
" (2 ") + guanine.....	1 "	2	92
" (2 ") + xanthine.....	1 "	8	74
" (2 ") + uracil.....	1 "	2	96
" (1 ") and xanthine (1 mg.) + adenine.....	1 "	4	82
" (1 ") " " (1 ") + guanine.....	1 "	4	86
" (1 ") " " (1 ") + uracil.....	1 "	2	79
Adenine, guanine, xanthine, uracil (1 mg. each) + solubilized liver.....	40 γ	4	89
Adenine, guanine, xanthine, uracil (1 mg. each) + solubilized liver.....	200 "	4	73

* G = galvanometer reading; 100 = no growth; 0 = total extinction of light; about 70 = maximum growth.

† Solubilized liver is a source of "folic acid."

From a study of the effect of purines and pyrimidines upon *Streptococcus lactis* R (Table I) it was found that these materials are active only in certain combinations. The most active combination was that of thymine with xanthine. These observations indicate that *Streptococcus lactis* R is stimulated by a pyrimidine and a purine. The pyrimidine may be thymine or a compound in liver, and the purine may be xanthine or a similar com-

pound. The large amounts of thymine (2 to 4 γ per tube) and xanthine (100 γ per tube) required suggest that these molecules are altered before they are physiologically active.

The addition of a third member to this combination decreased the amount of growth obtained, suggesting that purines other than xanthine which are present in the medium combine stoichiometrically with the pyrimidine component to form a molecule which is less active physiologically than the xanthine-thymine (or their derivative) compound. This effect is similar to that of *p*-aminobenzoic acid *versus* the sulfonamides (8). Pennington noted similar results while studying stimulatory compounds with *Spirillum serpens* (9).

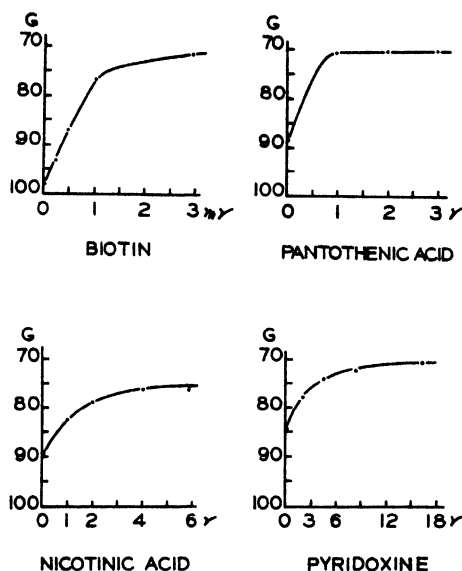


FIG. 2. Effect of vitamins on the growth of *Streptococcus lactis* R. Crystalline biotin acid was used for the biotin curve. G = galvanometer readings.

It is evident from these results that materials containing large amounts of thymine can be accurately assayed for "folic acid" only when extra precautions are taken. The amount of thymine in a sample could probably be estimated by determining growth activity after the sample has been treated with acid to destroy the "folic acid."

Vitamin Requirements—In order to determine the vitamin requirements of *Streptococcus lactis* R the vitamin to be studied was omitted from the basal medium (3). "Folic acid" was added to each tube in the form of a concentrate, the Super Filtrol eluate (1), equivalent to 100 to 500 γ of solubilized liver. Typical curves showing the quantitative requirements

of the vitamins found to be necessary for the growth of *S. lactis* R are given in Fig. 2. From these and similar experiments, the approximate minimum vitamin requirements for *S. lactis* R in 10 ml. of a casein hydrolysate-synthetic medium are estimated to be as follows: biotin 1 to 4 millimicrograms, calcium pantothenate <1 γ , nicotinic acid 1 to 4 γ , and pyridoxine 8 to 10 γ . Thiamine and riboflavin were without effect on this medium. *S. lactis* R has previously been reported to require 0.3 γ of pantothenic acid per 10 ml. by Snell, Strong, and Peterson (10) and up to 3 γ of pyridoxine per 10 ml. by Snell and Guirard (11).

These studies indicate that microbiological assays for biotin, pantothenic acid, nicotinic acid, and pyridoxine are possible with *Streptococcus lactis* R as the test organism.

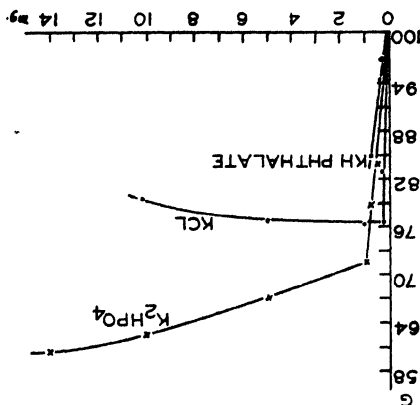


FIG. 3

FIG. 3. Growth-dosage curves of *Streptococcus lactis* R with potassium salts. G = galvanometer reading.

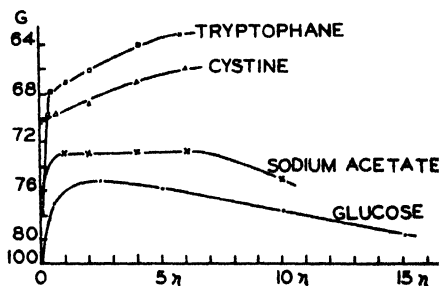


FIG. 4

FIG. 4. Effect of nutrients on the growth of *Streptococcus lactis* R. Tryptophane, 0.5 N; cystine, 1 N; sodium acetate, 10 N; glucose, 20 N. G = galvanometer reading.

Mineral Requirements—A similar set of experiments was devised to determine the mineral requirements of this bacterium. All of the salts were omitted from the medium for “folic acid” assay (with the exception that the medium was neutralized with 10 per cent sodium hydroxide), and Super Filtrol eluate equivalent to 200 γ of solubilized liver was added to each tube. With this medium, potassium was the only mineral that needed to be added to produce good growth. In the absence of added potassium no growth was obtained. The following salts were added and found to have no effect: NH_4Cl (0.1 to 10 mg.), NaCl (1 to 20 mg.), NaH_2PO_4 (0.25 to 10 mg.), MgNO_3 (0.05 to 2 mg.), FeSO_4 (2.5 to 100 γ), and MnSO_4 (2.5 to 100 γ). Experiments with potassium salts show (Fig. 3) that the

potassium in potassium chloride, dipotassium phosphate, and potassium acid phthalate is equally available. The increased amount of growth in the case of dipotassium phosphate above the actual potassium requirement (about 1 mg. of the salt), as compared to the decreased growth obtained by adding potassium chloride above the potassium requirement, may be accounted for by the buffering action of the dibasic salt.

These curves indicate that this organism might be used in a microbiological assay for potassium.

Requirements for Other Nutrients—The requirement for each of the other nutrients under the conditions of the assay was determined by the same technique. The results are summarized in Fig. 4. The best growth (as

TABLE II
Basal Medium for Streptococcus lactis R in 16 Hour "Folic Acid" Assay

Constituent	Amount per tube	Amount per 200 tubes
Sodium acetate.....	20 mg.	4 gm.
Glucose.....	100 "	20 "
Casein (acid-hydrolyzed).....	50 "	10 "
L-Cystine.....	1 "	200 mg.
L-Tryptophane.....	3 "	600 "
Adenine sulfate.....	0.1 mg.	20 "
Guanine hydrochloride.....	0.1 "	20 "
Xanthine.....	0.1 "	20 "
Thiamine hydrochloride.....	2 γ	0.4 mg.
Riboflavin.....	2 "	0.4 "
Nicotinic acid.....	6 "	1.2 "
Pyridoxine hydrochloride.....	12 "	2.4 "
Calcium pantothenate.....	4 "	0.8 "
Biotin (concentrate).....	4 millimi- crograms	0.8 γ
K ₂ HPO ₄	50 mg.	10 gm.
Water to make.....	5 cc.	1 liter

measured by turbidity) was obtained with 3 mg. of tryptophane, 1 to 3 mg. of cystine, 10 mg. of sodium acetate, and about 40 mg. of glucose per tube.

Medium—A complete basal medium for the "folic acid" assay based on the above results is given in Table II.

The solutions for the various constituents of the medium are made up as follows:

Vitamine-free, acid-hydrolyzed casein.³ 50 gm. of Labco casein are mixed with 500 ml. of HCl (280 ml. of concentrated acid diluted to 500 ml.) and refluxed for 48 hours. The hydrolysate is concentrated *in vacuo*

³ This procedure was adapted from that of Mitchell *et al.* (3).

to a thick syrup, redissolved in water, and reconcentrated four times. It is then taken up in water (500 ml.) neutralized to pH 3 with NH_4OH , and 5 gm. of norit A are added. The mixture is stirred for 30 minutes and filtered. The filtrate is neutralized with NH_4OH . This, as well as other biological solutions, is preserved with toluene and kept cold.

Adenine and guanine. These are dissolved together in a small amount of HCl with heat and diluted to 1 mg. in 4 ml. of water.

Xanthine is dissolved in NH_4OH and diluted to 1 mg. per ml.

Cystine and tryptophane. Each of these is dissolved in a small amount of HCl and diluted to 1 mg. per ml. and 10 mg. per ml. respectively.

Vitamins. 60 mg. of pyridoxine hydrochloride, 20 mg. of calcium pantothenate, 30 mg. of nicotinic acid, 10 mg. of thiamine hydrochloride, 10 mg. of riboflavin, and 20 γ of biotin (S. M. A. Corporation's concentrate No.

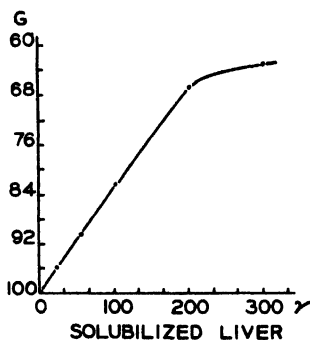


FIG. 5. Growth-dosage curve with *Streptococcus lactis* R on the new medium. G = galvanometer reading.

1000 may be used) are added to 100 ml. of water. This mixture is shaken well before being added to the basal medium (0.02 ml. per tube).

Base. 10 per cent KOH is used to neutralize the medium to pH 7.0 to 7.2.

This medium is more reliable because extra amounts of vitamins supplied by the material to be assayed have no effect on the growth produced. The increased buffering action gives about twice as much growth as did the medium of Mitchell and Snell. Standard curves obtained with this new medium give a wider assay range and have a linear relationship between the amount of solubilized liver added and the amount of growth produced. (Compare the curve in Fig. 5 with that obtained with the old medium in Fig. 1.) It seems doubtful that maximum growth is obtained, since titration rarely exceeds 4 cc. of 0.1 N NaOH per tube. Further evidence for this is obtained when large amounts of Super Filtrol eluate and solubilized liver are used to make growth-dosage curves. The curve obtained

with the eluate reaches a plateau, while the liver curve continues upward. It has not been determined whether this effect is due to an extra amount of the known vitamins, a buffering action, or an unknown factor present in the liver which is not present in the eluate.

In Table III typical results obtained with this medium are compared with those obtained with the old medium. Vitamin-rich materials show less "folic acid" activity with this medium than with the old one, since added vitamins have no effect on the growth obtained with the new medium.

Compounds Tested for "Folic Acid" Activity—The following compounds were tested qualitatively by placing the amount indicated in tubes containing the basal medium. None of the compounds showed any "folic acid" activity.

Amino acids which were tested for "folic acid" activity are β -alanine (50 to 500 γ), *l*-lysine (50 to 500 γ), *l*-leucine (0.05 to 1 mg.), *l*-aspartic acid

TABLE III
*Activity of Materials Used for "Folic Acid" Sources**

Material	Old media	New media	
	<i>Streptococcus lactis</i> R	<i>Streptococcus lactis</i> R	<i>Lactobacillus casei</i>
Solubilized liver.....	100	100	100
Whole liver substance.....	230	186	68
Liver Fraction B.....	106	98	
Grass juice powder.....	187	147	68
Dried grass extract powder.....		11	29

* Based upon the standard, solubilized liver, having 100 per cent activity.

(50 to 500 γ), *d*-glutamic acid (50 to 500 γ), *l*-arginine (50 to 500 γ), and glycine (10 to 500 γ).

Other compounds of biological importance which were tested are *i*-inositol (10 to 100 γ), choline chloride (10 to 100 γ), *l*-ascorbic acid (1 to 100 γ), 2-methyl-1,4-naphthoquinone (50 to 500 γ), urea (10 to 50 γ), uracil (50 to 500 γ), uric acid (1 to 100 γ), *d*-ribose (10 to 500 γ), orotic acid (1 to 50 γ), thymus nucleic acid (2 to 100 γ), calcium nteleinat (1 to 100 γ), xanthopterin⁴ (0.001 to 500 γ), and *p*-aminobenzoic acid (10 to 100 γ).

Compounds similar in structure to *p*-aminobenzoic acid which were tested are benzoic acid (10 to 100 γ), *o*-aminobenzoic acid (10 to 100 γ), *m*-aminobenzoic acid (0.5 to 200 γ), potassium acid phthalate (0.01 to 200 γ), *p*-aminodiphenyl (1 to 100 γ), *p*-aminophenyl (1 to 100 γ), *p*-aminophenylacetic acid (1 to 100 γ), *p*-aminophenylglycine dihydrochloride (1

⁴ Prepared by R. C. Mills.

to 100 γ), ethyl-*p*-aminobenzoate (0.01 to 200 γ), *p*-dimethylaminoazobenzene (10 to 100 γ), aniline (30 to 100 γ), salicylic acid (10 to 100 γ), acetanilide (10 to 100 γ), benzidine (10 to 100 γ), and *p*-aminoazobenzene (10 to 100 γ).

Miscellaneous compounds which were tested are 2,4-diamino-6-oxypyrimidine⁴ (10 to 100 γ), isonitro derivative of 2,4-diamino-6-oxypyrimidine⁴ (10 to 100 γ), 2,4,5-triamino-6-oxypyrimidine⁴ (10 to 100 γ), malonic ester (10 to 50 γ), sodium citrate (50 to 500 γ), pyridine (1 to 20 γ), guanidine (10 to 100 γ), and sodium diethyl barbiturate (10 to 100 γ). The inactivity of these compounds serves to increase the significance of the growth responses obtained with thymine and xanthine.

The "folic acid" content of a few materials used as a source of this factor was determined by means of both *Streptococcus lactis* R and *Lactobacillus casei* (Table III). The medium developed here was used; however, Speakman's salts (12) were added to the media for *Lactobacillus casei*, since it was found that good growth could not be obtained with this organism without large amounts of manganese or magnesium ions. When representative liver preparations used in study of the vitamin B complex on chicks (1) were assayed in the same fashion, the results with the two organisms agreed very closely.

DISCUSSION

"Folic acid," as previously mentioned, is the name applied to a factor necessary for the growth of *Streptococcus lactis* R (2). It was originally differentiated from the eluate factor (a factor described by Snell and Peterson (13) and later by Stokstad (7) as a factor necessary for *Lactobacillus casei*) by the fact that "folic acid" contained no phosphorus (3). This difference was eliminated when Stokstad announced that his purer concentrates were free from phosphorus (14). Mitchell *et al.* recognized that a "folic acid" concentrate produced growth of *Lactobacillus casei* under their conditions (3). Likewise Hutchings *et al.* found that the eluate factor was of general nutritional importance within the group of lactic acid bacteria and the growth of *Streptococcus lactis* R was stimulated by the eluate factor under their conditions (15).

Hutchings *et al.* (16) presented evidence suggesting that the *Lactobacillus casei* factor was identical with an unknown chick factor. We have since given evidence for the existence of at least three factors in norit eluate concentrates from liver; namely, vitamin B₁₀, vitamin B₁₁, and a factor needed by both *Lactobacillus casei* and *Streptococcus lactis* R (1).

Recently a chick antianemia factor, designated by Hogan and Parrott as vitamin B₆ (17), has been reported to have been isolated in crystalline form (18). Observations were made to "demonstrate conclusively the

identity of Hogan's antianemia factor and Peterson's 'eluate factor.'" However, Keresztesy, Rickes, and Stokes have isolated a compound which is active for *Streptococcus lactis* R but inactive for *Lactobacillus casei* (19). For reasons not divulged they indicate that this compound is neither "folic acid" nor the eluate factor.

Stokstad (14) has lately announced the isolation of a crystalline material which seems identical from chemical analysis with the material isolated by Piffner *et al.* (18). Stokstad's crystals are active for both bacteria when isolated from liver but they are only one-half as active for *Streptococcus lactis* R when isolated from yeast.

We have found (Table III) that whole liver substance and grass juice powder contain more of the growth substance (or substances) required by *Streptococcus lactis* R and relatively smaller amounts of the factor (or factors) required by *Lactobacillus casei* than does solubilized liver. This ratio is reversed in the case of grass extract powder. These observations and the above reports indicate the existence of at least two compounds which are physiologically active for these two bacteria.

SUMMARY

The optimum amounts of biotin, nicotinic acid, pantothenic acid, pyridoxine, dipotassium phosphate, sodium acetate, tryptophane, glucose, and certain purines and pyrimidines were determined for the growth of *Streptococcus lactis* R on a casein hydrolysate-synthetic medium in order to facilitate the use of this organism in assay work. The data were used to build a new medium for *Streptococcus lactis* R which may be used to give a more reliable "folic acid" assay.

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THE EFFECT OF pH OF THE FILTRATE ON THE RECOVERY OF INULIN AND DIODRAST FROM BLOOD PLASMA

By NATHAN W. SHOCK*

(From the Division of Chemotherapy, National Institute of Health, United States Public Health Service, Federal Security Agency, and the Baltimore City Hospitals, Baltimore)

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Removal of proteins from blood plasma by precipitation with cadmium sulfate and sodium hydroxide (3) has been recommended and used in the preparation of blood filtrates for the estimation of inulin and diodrast by previous investigators (1, 5, 8). Others have been unable to recover inulin quantitatively from such filtrates (6, 7). This paper presents data which throw some light on a probable source of these differences, and indicates that the pH of the filtrate influences the recovery of inulin.

EXPERIMENTAL

Samples of blood plasma containing 20 mg. of inulin and 2 mg. of diodrast per 100 cc. were prepared. 20 mg. of purified inulin were dissolved in 5 cc. of 0.9 per cent NaCl containing 2 mg. of diodrast and diluted to 100 cc. final volume with the plasma. Blood 1 was a citrated plasma obtained from a blood bank. Bloods 2 and 3 were drawn directly into dry flasks containing the required amount of dry heparin to prevent clotting. The blood was centrifuged and the plasma separated for use. Samples of each plasma were reserved without addition of inulin or diodrast for blank tests. Both control and experimental samples of plasma were treated with washed yeast for the removal of glucose prior to the preparation of filtrates. Filtrates of varying pH were prepared from each plasma by adding 1 volume of 0.80, 0.90, 1.00, 1.05, 1.10, 1.12, 1.15, or 1.20 N sodium hydroxide to 1 volume of plasma in 8 volumes of acid cadmium sulfate solution. Control or blank filtrates were prepared with each concentration of alkali as well. Thus filtrates were obtained at eight different pH levels for each blood. After filtration, the pH of the filtrate was immediately determined with the glass electrode.

Inulin was determined in each filtrate by the following modification of Harrison's method (4): to 5 cc. of filtrate, diluted to contain from 3 to 20 γ of inulin per cc., 10 cc. of diphenylamine reagent were added in a 19 $\times$ 150 mm. Pyrex test-tube graduated to contain 15 cc. (Fresh diphenylamine reagent was made each day by dissolving 3 gm. of diphenylamine (Merck's) in 100 cc. of glacial acetic acid. To this solution 60 cc. of con-

* With the technical assistance of Marvin J. Yiengst.

centrated HCl were added.) After the contents of the tubes were mixed well, the tubes were heated in a boiling water bath for 60 minutes, cooled to room temperature in tap water, and the volume adjusted to the 15 cc. mark by the addition of water. After the mixing, the color was compared with a water blank similarly treated, at a wave-length of $640\text{ m}\mu$ in the Coleman universal spectrophotometer with a No. PC-4 filter and a slit width of $35\text{ m}\mu$. Blank determinations were made on plasma filtrates prepared at the same pH.

Diodrast was determined in each filtrate according to the method of Flox, Pitesky, and Alving (2). The color intensity of the iodine liberated

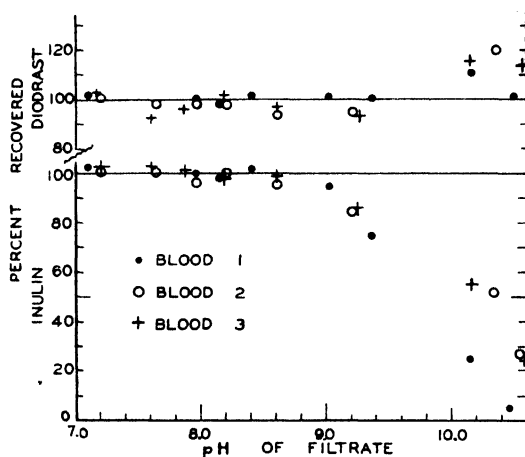


Fig. 1. Effect of changes in pH of plasma filtrates on the recovery of added inulin and diodrast.

after 10 minutes was read in the Coleman universal spectrophotometer at a wave-length of $400\text{ m}\mu$ with a No. PC-6 filter.

Results

The results obtained with three blood samples are shown in Fig. 1, in which the pH of the filtrate is plotted against the per cent of inulin or diodrast which was recovered. The data indicate that for complete recovery of inulin the pH of the filtrate must be less than 8.5. No loss of diodrast was observed, but in filtrates with pH greater than 9.5, the apparent recovery was greater than 100 per cent.

SUMMARY

The recovery of inulin from blood plasma filtrates prepared by the precipitation of cadmium sulfate with sodium hydroxide was incomplete in

filtrates with a pH greater than 8.4. At pH 10.2, less than 5 per cent of the added inulin was recovered. Recovery of diodrast was complete in filtrates with a pH of 9.5 or less. At higher pH levels, high diodrast readings were obtained.

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THE ADRENALS AND THE MOBILIZATION OF STORED FAT FORMED FROM DIETS CONTAINING DIFFERENT FATS*

BY LEO T. SAMUELS AND ROBERT F. CONANT

(From the Division of Physiological Chemistry, Department of Physiology, University of Minnesota Medical School, Minneapolis)

(Received for publication, September 14, 1943)

It has been amply demonstrated that the metabolism of the rat is disturbed by adrenalectomy. The more rapid disappearance of carbohydrate on fasting has been attributed to decreased glycconeogenesis from protein (1, 2). However, the accumulation of fat in the liver is also decreased and is not restored to normal by salts alone (3, 4). Since a large part of the normal metabolism of the animal during fasting is that of fat, it was thought worth while to investigate the rate at which fat disappeared from the liver, mesenteric stores, and skeletal muscle of adrenalectomized rats when food was withheld.

Since tung oil contains a high amount of conjugated fatty acids, one diet contained this fat in an emulsified form, the original plan being to use this as a tracer. This did not prove feasible, but it was found that the presence of this fat considerably increased the excretion of acetone bodies and the loss of fat from the liver during fasting.

Methods

Young male rats of the Sprague-Dawley strain, 75 to 90 days old, were divided into twelve groups of five to eight rats each with similar distributions of body weight. They were then fed one of the two diets listed in Table I for 6 days by the stomach tube method of Reinecke *et al.* (5).

The sodium bicarbonate was included to increase the ketogenic effect of the diet. Each rat was given 1 cc. of diet twice a day for every 60 sq. cm. of body surface. All animals received the Rubin-Krick solution to drink (6). Two groups were adrenalectomized at the beginning of the feeding period, the lumbar approach being used, and four other groups at the end of the 6 day feeding period. Table II gives an outline of the treatment for each group.

Groups 1, 2, 5, and 6 were placed in metabolism cages during the feeding

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Assistance in the preparation of these materials was furnished by the personnel of the Work Projects Administration, Official Project No. 165-1-71-124, Sub-project No. 355.

period. The urine was collected in 48 hour portions and analyzed for total acetone bodies by the method of Van Slyke (7). During the fasting period the urine of Groups 11 and 12 was collected and analyzed in the same

TABLE I
Composition of Diets

The diets were mixed and run twice through a small manually operated homogenizer.

	Diet 1	Diet 2
35 per cent cream, <i>gm.</i>	17.6	69.2
57 " " " "	74.6	
Butter fat, <i>gm.</i>		30.8
Tung oil, <i>gm.</i>	7.8	
Vitamin B concentrate (Abbott), <i>capsule</i>	1	1
" D " (850,000 units per cc), <i>drop</i>	1	1
NaHCO ₃ , <i>gm.</i>	2	2
Fat (analysis), <i>per cent</i>	56.5	55
Tung oil fat, <i>per cent</i>	7.75	0

TABLE II
Urine Collection (2 Day Periods) during Experiments Covering 6 Days of Feeding Followed by 6 Days of Fasting

Group No	Diet	Treatment	Duration of urine collection
			<i>days</i>
1	Butter fat	Controls	6
2	" "	Adrenalectomized at beginning of feeding period	6
3	" "	Controls	12
4	" "	Adrenalectomized at beginning of fasting period	12
5	Tung oil	Controls	6
6	" "	Adrenalectomized at beginning of feeding period	6
7	" "	Controls	8
8	" "	Adrenalectomized at beginning of fasting period	8
9	" "	Controls	10
10	" "	Adrenalectomized at beginning of fasting period	10
11	" "	Controls	12
12	" "	Adrenalectomized at beginning of fasting period	12

manner. All values for total acetone bodies are expressed as the equivalent weight of acetone.

The animals were killed at the time indicated in Table II, and the livers were digested with 6 M KOH solution and the fatty acids extracted, titrated, and weighed as previously described (8). In Groups 5 to 12 the perirenal,

pararenal, and gonadal fat depots were also dissected out, dried with anhydrous Na_2SO_4 , ground, and extracted with petroleum ether. Aliquots of the petroleum ether extracts were then dried and weighed. The muscles of the thighs were also removed in these groups, dissected free of observable fat, and treated in the same manner as the fat depots.

Whenever adrenalectomy had been performed, the region around the upper pole of the kidney was examined for adrenal tissue. If any tissue resembled cortical tissue, the area was removed and examined under a dissecting microscope. In two cases tissue of possible cortical character was observed and these rats were discarded. Data for all other rats are included except when samples were lost during analysis.

Results

The excretion of acetone bodies is indicated in Fig. 1. The ketosis was relatively low on the diet in which the only source of fat other than the

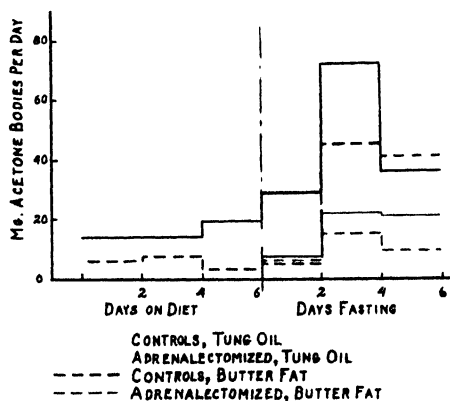


FIG. 1. Excretion of ketone bodies in the urine of normal and adrenalectomized male rats during fasting subsequent to diets containing different fat mixtures. Adrenalectomy was performed at the beginning of the fasting period.

vitamin concentrates was butter fat, even though sodium bicarbonate was administered. The effect of the presence of tung oil as 13.7 per cent of the total fat was quite marked. There was a 3-fold greater excretion of acetone bodies.

Fig. 1 also illustrates the effect of adrenalectomy, and of tung oil in the diet, on the ketosis during a subsequent period of fasting. The adrenalectomized rats excreted only about one-third of the acetone bodies excreted by the controls. They showed a similar curve of excretion during fasting, however. There was a rise during the 3rd and 4th days and a subsequent drop, but not to the level of the first 2 days of fasting.

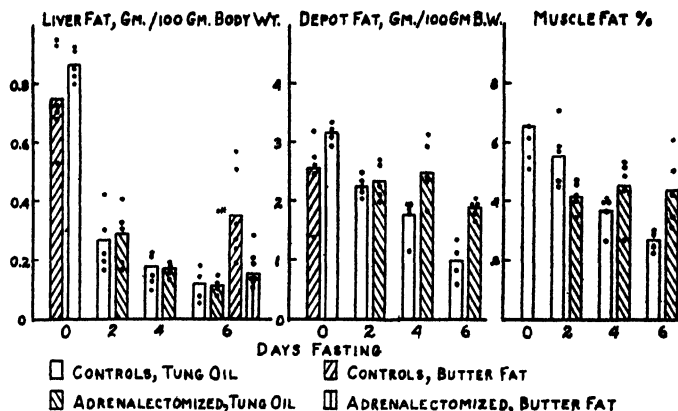


FIG. 2. Effect of fasting on the amount of liver, pararenal and gonadal depot, and muscle fat in normal and adrenalectomized male rats. The bars represent the mean values; the dots represent individual analyses. The value marked with an asterisk was not used in computing the mean.

TABLE III
Fat Content of Organs of Rats with and without Adrenals during Fasting Period

Group No.	Time fasted days	Fat in diet	Liver fat per 100 gm. body weight		Depot fat per 100 gm. body weight		Muscle fat	
			No. of rats	Average and standard error	No. of rats	Average and standard error	No. of rats	Average and standard error
1. Original	0	Butter	6	0.75 $\pm$ 0.065	5	2.56 $\pm$ 0.25		
2. " "	0	Tung oil	5	0.86 $\pm$ 0.024	4	3.15 $\pm$ 0.08	5	6.4 $\pm$ 0.67
3. Controls	2	" "	5	0.27 $\pm$ 0.043	5	2.26 $\pm$ 0.08	5	5.6 $\pm$ 0.47
4. Adrenalectomized	2	" "	5	0.28 $\pm$ 0.043	5	2.33 $\pm$ 0.14	5	4.2 $\pm$ 0.22
5. Controls	4	" "	5	0.16 $\pm$ 0.024	5	1.74 $\pm$ 0.15	5	3.7 $\pm$ 0.30
6. Adrenalectomized	4	" "	4	0.165 $\pm$ 0.013	5	2.55 $\pm$ 0.23	5	4.5 $\pm$ 0.48
7. Controls	6	" "	4	0.116 $\pm$ 0.029	4	0.99 $\pm$ 0.17	4	2.7 $\pm$ 0.14
8. Adrenalectomized	6	" "	4	0.115 $\pm$ 0.015	5	1.88 $\pm$ 0.07	5	4.4 $\pm$ 0.54
9. Controls	6	Butter	6	0.35 $\pm$ 0.063				
10. Adrenalectomized	6	" "	7	0.17 $\pm$ 0.024				

The effect of the presence of the tung oil in the diet persisted during the subsequent fasting period. Its marked tendency to increase the degree of ketosis was observable in the absence of the adrenals, and was therefore not

mediated through them. The period of highest excretion was not significantly prolonged, however; it was reached on the 3rd and 4th day and was followed by a drop in the subsequent 2 day period.

Fig. 2 and Table III show the change in fat content of the liver, fat depots, and muscles both after adrenalectomy and on the two types of fat diet. The removal of the adrenals did not significantly change the rate at which the fat content of the liver fell during fasting when the animal had previously been on the tung oil diet. When the previous diet had contained only butter fat, however, the decrease in liver fat was less in the controls than in the groups on tung oil. The difference was highly significant ($P = < 0.01$ for Groups 7 and 9 and Groups 8 and 9). Removal of the adrenals appeared to have accelerated the decrease until it was approximately the same as in the animals fed tung oil. The probability that the difference between the means for the control and adrenalectomized rats on the butter fat diet was due to chance was 0.02.

Removal of the adrenals did affect the rate at which fat disappeared from the fat depots and muscles, however. The change during the first 2 days after the operation was similar in both groups, probably because of the effect of circulating hormone. After this, however, the decrease was slow and irregular in the adrenalectomized group, while the controls continued to use fat at a steady rate. The net utilization of stored fat during fasting seemed to be accelerated by the presence of the adrenal glands. The differences by the 6th day were highly significant: for muscle fat $P = 0.02$ and for depot fat $P = < 0.01$.

During the feeding period, adrenalectomized rats accumulated only half as much fat in the livers as did the controls. This was true on either the tung oil or butter fat diets.

DISCUSSION

It would seem that the results obtained in this study could be best explained by two assumptions: that adrenalectomy decreased the rate of transport of fat from the body stores to the liver or its uptake by the liver, and that the presence of tung oil increased the rate of fat metabolism in the liver.

The lipolytic action of the tung oil would explain the increased ketosis in the normal animals on this diet and in subsequent fasting, associated with a more rapid drop in both liver and depot fat while fasting.

On this basis the ketosis was reduced in the adrenalectomized rats, because the fat was not removed by the liver with sufficient rapidity to enable it to form acetone bodies at the normal rate. This is borne out by the slower fall in depot fat during fasting. The lower level of liver fat during feeding with either diet and the more rapid decrease in liver fat

during fasting in the adrenalectomized rats fed butter fat are also compatible with this view. Barnes *et al.* (9) have shown that conjugated fatty acids in the neutral fat fed did not accumulate as rapidly in the livers of adrenalectomized rats as in the normals, although they appeared with normal rapidity in the phospholipid fraction. In cats, Yeakel and Blanchard (10) report lower plasma lipids after adrenalectomy. This would agree with the general thesis that adrenalectomy interferes with the passage of neutral fats (*e.g.* depot fat) into the hepatic cells and thus lowers the fat available for metabolism by the liver.

If this is true, however, why did not the liver fat in the adrenalectomized rats on the tung oil diet fall more rapidly than that of the controls during fasting? One explanation would be that the fats were being metabolized so rapidly after the tung oil diet that the rate of fall of the liver fat was maximal. When one considers that the fat content of the livers fell from an average of 0.9 gm. per 100 gm. of body weight to 0.1 gm. per 100 gm. of body weight in a period of 6 days during which fat mobilization and utilization was rapid, it is obvious that fat was passing out of the liver at a high rate. This is confirmed by the average acetone excretion of 71.5 mg. per day for a 150 gm. rat. In the adrenalectomized rats previously on the butter fat diet this rate of disappearance was approached but was not quite reached. If, then, this represents the maximal rate of fat disappearance under the circumstances, it is understandable that the adrenalectomized rats did not lose fat from the livers at any greater rate. The difference due to adrenalectomy should be reflected, rather, in a greater difference in acetone body excretion. This appears to be the case; the excretion in the animals without adrenals is one-third that of the controls when both have previously been on a butter fat diet, while it is only about one-fourth that of the animals on the tung oil.

Verzár and Laszt (11) and Bavetta and Deuel (12) have introduced evidence favoring a delayed absorption of fatty acids as an important factor in fat metabolism of the adrenalectomized rat. While there may have been a small decrease in absorption, it is doubtful whether this could be a significant factor in the differences in liver fat and acetone body excretion, since the differences were similar during feeding and when adrenalectomy was performed after the last food was fed; in the latter case absorption could not possibly be the causal factor.

SUMMARY

Male rats 75 to 90 days of age were fed for 6 days on high fat diets, containing either almost all butter fat or 17 per cent tung oil and 73 per cent butter fat. They were then fasted for 6 days. Some rats were adrenalectomized at the beginning of feeding and some at the start of the fasting

period. Groups of both adrenalectomized and control rats were killed at the end of feeding and at intervals during the fasting period.

The presence of tung oil in the diet increased the acetone body excretion in all animals both during feeding and in the subsequent fasting period.

On fasting the ketosis increased, reaching a peak on the 3rd and 4th days.

The ketosis was greater when the rats had been on a tung oil diet if normal or adrenalectomized rats were compared with similar rats fed butter fat only.

The acetone body excretion was always greater in the normal fasting rats than in adrenalectomized rats on the same diet.

The liver fats of both adrenalectomized and control rats on the tung oil diet fell rapidly during fasting. The adrenalectomized rats used up their depot fats at a much slower rate than the controls, however. The liver fats of control rats which had been on a straight butter fat diet did not decrease as rapidly on fasting as in the adrenalectomized rats or the rats previously on the tung oil diet.

An explanation of the data is offered on the assumption that the tung oil primarily affected the breakdown of fat in, and transport from, the liver, while adrenalectomy primarily affected the transport from the depots to the liver.

Since the same type of differences appeared in fasting between controls and rats adrenalectomized after the last feeding as occurred between the two types of rats during feeding, it seems unlikely that changes in fat absorption were major factors in the differences in liver fat and acetone body excretion.

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STUDIES ON THE NUTRITIONAL BASIS OF ABNORMAL BEHAVIOR IN ALBINO RATS

IV. CONVULSIVE SEIZURES ASSOCIATED WITH PYRIDOXINE DEFICIENCY*

By R. A. PATTON, H. W. KARN, AND HERBERT E. LONGENECKER

*(From the Department of Psychology and the Department of Chemistry, University of
Pittsburgh, Pittsburgh)*

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The rat has been observed to exhibit a behavior pattern characterized by violent convulsive seizures when exposed to intense auditory stimulation. Such seizures were first observed by Maier (22) when rats were forced to respond to a difficult problem. "Conflict" imposed by forced reaction in a situation involving an insolvable problem was at first thought to be a necessary determinant of the abnormal behavior (23). Subsequent studies (1, 28, 31), however, demonstrated clearly that auditory stimulation alone was an effective means of eliciting such convulsions and, in addition (29), that the existence of a problem was not of basic importance for the appearance of the seizures.

In previous studies in our laboratories, Patton, Karn, and King (32-34) obtained evidence that nutritional factors play an important rôle in the susceptibility of the rat to convulsive seizures induced by exposure to sound. Furthermore, inanition, vitamin B₁, and vitamin B complex deficiencies were found to be contributing factors to increased sensitivity. A significant finding of one of these studies (34) was the fact that such convulsions, associated with low food intake and persisting despite high vitamin B₁ supplements, could be quickly reduced by the addition of a group of vitamin and mineral supplements. Reduction in sensitivity occurred even with a low caloric intake. Thus, there was the indication that protection was afforded either by the supplements *per se*, or that they permitted a more efficient utilization of food substances already present in the reduced food allowance.

A continuation of the work has entailed the investigation of the specific or combined functions of individual components of the vitamin B complex. In the present experiments the effects of pyridoxine deficiency have been studied.

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A number of investigators have reported the occurrence of a convulsive state in experimental animals subjected to pyridoxine deficiencies. Chick, El Sadr, and Worden (2) found that fits of an epileptiform nature appeared in rats maintained for long periods on a diet deficient in vitamin B₆. The seizures were characterized by hyperexcitability and circular running, tonic-clonic convulsions, and a comatose recovery period. Daniel, Kline, and Tolle (6) found that similar seizures appeared in young rats while being nursed by mothers maintained on pyridoxine-deficient diets. Although the diets were satisfactory from the standpoint of growth and reproduction, convulsions and failure of the young appeared suddenly toward the end of the lactation period. Such symptoms could be cured or prevented by pyridoxine supplements. Hyperirritability and convulsions accompanying pyridoxine deficiencies have been observed by other investigators in the pig (3, 39, 40), the chick (19), and in the dog (11). In all of these studies auditory stimuli have been found capable of precipitating attacks.

The present experiments were designed primarily to investigate possible relationships existing between the sound-induced seizures studied previously by Patton, Karn, and King and the convulsive fits referred to above which accompany pyridoxine deficiency in the rat. As a technique to impose varying degrees of pyridoxine deficiency upon young animals, the mothers at parturition were placed on pyridoxine-free synthetic diets or on diets having this vitamin present in graded amounts. Seizures which ranged from fatal spontaneous convulsions through a less acute convulsive state capable of being repeatedly elicited by exposure to sound have been observed. A decreasing sensitivity to sound-induced seizures has been correlated with increased dietary levels of pyridoxine.

EXPERIMENTAL

The rats used in these experiments were select male and female breeders¹ with relatively uniform genetic and dietary backgrounds. The females were approximately 100 days old when procured and averaged 220 to 250 gm. in weight. When pregnant, the females were transferred to individual cages with raised, screen bottoms to prevent refection. Purina dog chow constituted the diet through pregnancy until shortly before parturition, when synthetic diets (see below) were used. Litters were reduced to six on the 2nd day after birth and were regularly weaned at 21 days of age.

Two basal experimental diets, essentially free of pyridoxine, were used throughout this work. The major components of these diets (in gm. per 100 gm.) were sucrose 71, casein (S. M. A. vitamin test) 18, fat (Crisco) 5, salt mixture (6) 4, and cod liver oil (Mead's) 2. To each 20 gm. of this

¹ Sprague-Dawley, Inc., Madison, Wisconsin.

mixture were added choline and two levels of thiamine, riboflavin, and pantothenic acid ^{2, 3} to complete basal Diet I and basal Diet II, as given in the accompanying tabulation. The thiamine, riboflavin, and pantothenic

	Diet I	Diet II
Thiamine, γ	40	150
Riboflavin, γ	60	150
Pantothenic acid, γ	100	200
Choline, mg.....	10	10

acid content of Diet I was comparable to that employed by Daniel, Kline, and Tolle (6) who found that complete protection against spontaneous seizures was afforded by 40 γ of pyridoxine per day. The amounts of pyridoxine provided in our experiments are shown in Table I.

TABLE I
Amounts of Pyridoxine Hydrochloride Supplied per 20 Gm. of Diet

Diet No.	Pyridoxine	Diet No.	Pyridoxine
	γ		γ
Ia	0	IIa	0
Ib	25	IIb	25
Ic	50	IIc	50
		IId	100
		IIe	150

Auditory Tests—All auditory tests were given in a sound-proofed box containing a resonated buzzer. (This apparatus has been previously described (30).) A standard exposure of 2 minutes was used for all tests.⁴ Five consecutive daily tests were given the females prior to breeding. No trace of sensitivity appeared in any animal during these tests. Three similar tests were again made on all females when the young were weaned. Unless otherwise specified all young animals were given standard tests every other day beginning at the 17th day.

Pyridoxine-Deficient Diets—Spontaneous convulsions occurred in young rats suckled by mothers maintained from parturition on the pyridoxine-free diets (Nos. Ia and IIa). 63 young rats from eleven different litters were

² The vitamins used in these experiments were generously provided by Merck and Company, Inc., Rahway, New Jersey.

³ Diets were made up in 2 kilo lots. Vitamins in the amounts required were first mixed with the casein for 2 hours in a ball mill, after which the sucrose and salts were added and the total mixed for 3 additional hours.

⁴ For detailed investigations of the essential characteristics of auditory stimuli capable of precipitating seizures, see Morgan and Galambos (27) and Galambos and Morgan (12).

studied (Table II). Spontaneous seizures were observed in forty-nine cases but undoubtedly occurred in many other cases at times when no one was in the laboratory.

There was some variation in the age at which spontaneous convulsions were first observed, but the majority occurred between the 17th and 19th days. Initial symptoms included tremors, chewing movements, face washing, and retraction of the head with extension of the fore legs. Some hours later these animals showed bursts of circular running or crawling,

TABLE II
*Convulsive Seizures in Pyridoxine-Deficient Young Rats and Effects of
Pyridoxine Supplements*

Diet No.	Litter No.	No. in litter	Age at which convulsions were observed	Supplemented with pyridoxine*			Not supplemented with pyridoxine	
				No. of animals	Amount of pyridoxine	No. of survivals 40 days after starting supplement	No. of animals	Age at which last death occurred in group
			days		γ			days
Ia	1	6	19-23	3	10	2	3	28
"	2	6	18-20	2	10	0	4	22
"	3	6	17-19	2	10	2	4	20
IIa	4	5	15-20				5	21
"	5	6	14-18	3	10	3	3	20
"	6	6	12-15				6	17
"	7	6	13-15	3	10	1	3	17
"	8	6	18-21	2	50	2	4	21
"	9	6	18-20	3	50	3	3	23
"	10	5	11-13				5	15
"	11	5	12-15	3	50	2	2	17
Total.....		63		21		15		

* The supplement (pyridoxine hydrochloride in 0.1 ml. of distilled water) was administered daily by pipette immediately after convulsive symptoms were first observed.

gave loud cries, and had convulsions. Seizures recurred at shorter and shorter intervals, with death generally ensuing on the following day. Stimuli of various types, such as sounds, a puff of air, or handling, were all effective in inducing spasms in these animals after the initial symptoms were noticed.

Litters 4, 5, and 6 (*cf.* Table II) were subjected to the standard auditory tests immediately after the initial spontaneous symptoms of sensitivity were observed. Out of sixteen animals in which spontaneous seizures were later observed, nine convulsions were elicited immediately by the buzzer. Of

the seven remaining animals, four showed hyperactivity in response to the buzzer and three of these developed severe convulsions approximately 5 minutes after being returned to their cages. In Litter 7, auditory tests were given from the 10th day after birth. These tests were ineffective in producing convulsions until after the initial symptoms (tremors, etc.) had appeared spontaneously.

No convulsive seizures were observed in any of the mothers during this time, nor was there any sign of sensitivity in the mothers during three additional sound tests given 21 days after parturition.

It is interesting to note that fewer spontaneous seizures were observed in litters from mothers which had been maintained on Diet Ia (less thiamine, riboflavin, and pantothenic acid content than in Diet IIa). These litters also appeared less well nourished than those on Diet IIa. Records of the food intakes of the lactating mothers receiving Diets Ia and IIa showed great individual variations, but in general there was a decreased consumption of food during the lactation period, from an average of 21.5 gm. at parturition to approximately 16 gm. when the young were weaned.

Effect of Pyridoxine Supplements on Sound-Induced Convulsions—As shown in Table II, pyridoxine supplements in the amounts of 10 and 50 γ of pyridoxine hydrochloride daily were effective in alleviating convulsions in eleven out of fourteen young on Diet IIa, and four out of seven on Diet Ia. The convulsions stopped in most of the supplemented animals as suddenly as they had appeared.

To determine the continued effect of pyridoxine on sensitivity to sound, these animals were maintained on their respective diets and supplements until they were 60 days of age. From weaning at 21 days, auditory tests were given every other day, resulting in a total of twenty tests for each animal.

The total incidence of seizures throughout the 40 day observation period is shown in Table III. It is evident that, although spontaneous seizures were alleviated by pyridoxine, complete protection did not seem to be afforded against exposure to sound. Most of these animals showed a high and continued level of sound-induced seizures with the convulsive type predominating. Two animals on Diet IIa showed an abrupt cessation of seizures and no further signs of sensitivity during these tests.

Incidence of Sound-Induced Seizures in Rats Maintained on Different Levels of Pyridoxine—In the next experiment, 147 young rats from twenty-five litters were observed during periods from just before weaning to 60 to 90 days of age. The litters raised on Diets Ib, Ic, and IIb were subnormal in weight and appearance at weaning. Also, weight gains of all six groups were subnormal during the experimental periods. Regular auditory tests, given every other day, were begun on 17 day-old suckling rats and discon-

tinued when it seemed that a stable level of sensitivity had been reached or, as in those groups receiving Diets Ib and Ic, an increasing number of deaths made further group comparisons difficult. No spontaneous convulsions were observed in any of these young animals, but sound-induced seizures were observed in all six groups (Table IV). The incidence of sensitivity for the groups raised on Diets IIb, IIc, IId, and IIe is shown in Fig. 1.

Significant aspects of the seizure curves of these animals include the levels of sensitivity reached, the elapsed time before maximum sensitivity

TABLE III
Effect of Pyridoxine Supplements on Sound-Induced Convulsions

Diet No.	No. of animals	Pyridoxine supplement	Total No. of tests	Effects of exposure to sound			Proportion of total observations		
				Convulsive seizures	Running attacks	No effect	Convulsive seizures	Running attacks	No effect
		γ					<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Ia	4	10	80	34	27	19	42	34	24
IIa	4	10	80	21	10	49	26	13	61
"	7	50	140	73	49	18	52	35	13

TABLE IV
Incidence and Type of Sound-Induced Seizures in Groups of Young Rats Maintained on Diets Containing Different Levels of Pyridoxine

Diet No.	Pyridoxine per 20 gm. diet	No. of litters	No. of animals	Average weight at 21 days	Total No. of seizures	Type of sound-induced seizure	
						Convulsive	Running
	γ			<i>gm.</i>		<i>per cent</i>	<i>per cent</i>
Ib	25	4	24	26	333	31	69
Ic	50	3	18	28	193	35	65
IIb	25	4	24	27	230	48	52
IIc	50	4	24	31	169	41	59
IId	100	5	28	30	99	6	94
IId	150	5	29	30	130	9	91

was attained, and the severity of attack (convulsion or hyperactivity) characteristically shown by a group.

Three additional daily tests were given to all the mothers beginning 21 days after parturition. These tests revealed signs of sensitivity (running) in only one animal (on Diet Ib). The average daily food intake in the mothers at the beginning of the lactation period was 20.5 gm. With a number of individual variations, the average daily food intake decreased to 16 gm. for groups on Diets Ib and Ic, 16.5 gm. for groups on Diets IIb and IIc, and approximately 19 gm. for those on Diets IId and IIe.

A high level of sensitivity developed rapidly in animals receiving Diets IIb and IIc. The development of sensitivity in these animals followed a similar sequence with seizures at first consisting only of short bursts of circular running. A steadily increasing number of convulsions emphasized the increasing severity of the attacks. This trend was well illustrated by the results of the last three tests given to those animals on Diet IIb. All the seizures observed during these trials were severe convulsive attacks.

The course of sensitivity observed in the above groups contrasts with that for animals receiving Diets Ib and Ic. It will be recalled that the latter diets included lower levels of thiamine, riboflavin, and pantothenic acid but the same levels of pyridoxine supplied by Diets IIb and IIc. Along with a less satisfactory gain in weight and general appearance, seizures in the

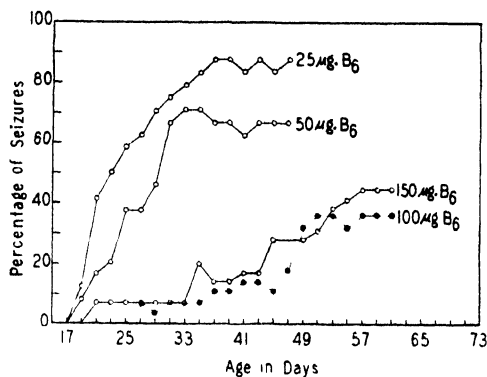


FIG. 1. Incidence of sound-induced seizures

animals receiving Diets Ib and Ic tended to be less severe, with running attacks predominating. When the highest level of sensitivity for a single day on Diet Ib was 66 per cent of the total number of animals, a higher percentage (79 per cent) of the twenty-four animals in the group showed seizures at some time during the experimental period. An increasing number of deaths from undetermined causes occurred in these two groups during the latter part of the testing period.

In groups receiving Diets IIId and IIe (higher levels of pyridoxine) sensitivity was delayed for significantly longer periods of time. Very few convulsions were noted (Table IV) and the attacks consisted only of short bursts of running. In two of the litters maintained on Diet IIe, there were three animals which showed scattered signs of sensitivity. The litters were from females which had produced convulsive young at an earlier breeding (Litters 4 and 5) when maintained on pyridoxine-free Diet IIa.

DISCUSSION

The spontaneous seizures observed during the course of these experiments seemed identical with the convulsive symptoms previously found by Daniel, Kline, and Tolle (6) to be associated with pyridoxine deficiency. In addition, it appears that the sound-induced convulsive seizures found associated with varying dietary levels of pyridoxine are identical with those described in previous studies by Patton, Karn, and King (32-34). A continuum of sensitivity ranging from spontaneous and eventually fatal seizures through a subacute convulsive state easily precipitated by sound has thus been found to be correlated with increasing levels of pyridoxine.

The present experimental findings seem to indicate that such an observed correlation does not necessarily imply a direct cause and effect relationship. In our first experiments, it was found that, when Diets Ia and IIa were supplemented with pyridoxine, the acute symptoms (spontaneous convulsions) rapidly disappeared and most of the young animals survived. In the second experiment, however, it appeared that such supplements given after the acute symptoms had begun were not effective in providing continued protection against exposure to sound.⁵ Later, the results again indicated a high degree of sensitivity to be associated with low levels of pyridoxine. A steadily increasing severity of attack was noted in animals receiving Diets Ib and IIb. With higher levels of pyridoxine a greater degree of protection was afforded. However, even in animals receiving Diets IId and IIe, sound-induced seizures did appear, although they were delayed and consistently less severe.

There is thus some indication that dietary factors other than a specific deficiency of pyridoxine alone may be of importance in the etiology of the seizures. Other necessary food constituents may not have been present in optimum amounts in the synthetic diets used or their functions may have been impaired by the lower levels of pyridoxine intake.

Strikingly similar convulsive seizures have been found to be associated with magnesium deficiency (15, 16, 20, 37). The extreme vasodilatation of the exposed body surfaces which was found to be coincident with hyperirritability and convulsions in magnesium-deficient rats has not been observed in any of our animals. It is of interest for the present problem, however,

⁵ There is the possibility that certain irreparable damage had resulted from the low level of pyridoxine intake or from the seizures themselves. These young animals in convulsion were frequently observed to be cyanotic. The arrest of breathing and symptoms of oxygen lack are well known in older susceptible animals (9, 30). There is evidence in the literature (13, 36, 38) that irreversible and summated damage to the central nervous system results in experimental animals repeatedly subjected to short periods of anoxia. Additional experimental work on the relation of oxygen lack to the seizures seems indicated.

that there is evidence (14) of a synergistic relation between a lack of magnesium and a lack of certain members of the vitamin B complex.

Recent studies of sound-induced seizures have emphasized the physiological aspects of the problem. Evidence has been presented concerning autonomic discharge (1, 9, 17, 18, 21, 24, 25, 30), blood changes following seizures (5), and the action of drugs in alleviating seizures (4, 17). Farris and Yeakel (7, 8) and Finger (10) have observed that age seems to be correlated with sensitivity.⁶ Lindsley, Finger, and Henry (21) found phases of the attack to be associated with abnormalities of the electroencephalogram similar to those observed during the epileptic seizure.

Apparently, a finely balanced mechanism is represented by the seizure threshold which may be influenced by a number of factors. It is to be expected that differences in the testing environment such as variations in the type of auditory stimulation or conflict-producing situations would be capable of modifying the susceptibility of an experimental animal. The results of the present experiments indicate that before these effects may be properly interpreted, the basic physiological factors underlying the seizures must be more fully investigated. There is also the indication that the results of these experiments may be applicable to the early detection and evaluation of marginal deficiencies when physiological injury has occurred without external evidence of malnutrition. Such studies offer a new approach to an understanding of the mechanism of convulsive seizures.

SUMMARY

Spontaneous convulsive seizures have been observed in young rats suckling from mothers maintained since parturition on synthetic diets deficient in pyridoxine but supplemented with thiamine, riboflavin, pantothenic acid, and choline. Such symptoms which appeared towards the end of lactation could be quickly alleviated by the administration of pyridoxine in amounts as low as 10 γ per day. However, neither 10 nor 50 γ of pyridoxine per day gave continued protection against similar convulsive seiz-

⁶ Recent studies (26, 35) appear to have demonstrated that the intestinal flora of the rat is capable of synthesizing in varying degrees many vitamins in the B complex. It is of interest that the cecum of young rats (where such bacterial synthesis is thought to occur) does not reach its maximum capacity until after the rat has passed 100 gm. in weight. At weaning, the cecum is only one-fourth as large relative to the digestive tract as it is in the adult animal. In the opinion of the above investigators this may account for the well recognized fact that rats at this stage are less resistant to some vitamin deficiencies than they are later. Coupled with the added requirements during a period of active growth, these facts may have a bearing on the above findings of Farris and Yeakel that there is a high incidence of seizures in young animals and that such susceptibility decreases with advancing age.

ures which regularly appeared when these animals were given standard auditory tests over a 40 day period.

When pyridoxine was included in the mother's diet at levels varying from 25 to 150 γ per day, no spontaneous seizures were observed in the young. However, a high incidence of sound-induced convulsive seizures was found when these young animals were given regular auditory tests from weaning at 21 days until they were from 45 to 90 days of age. No level of pyridoxine, from 25 γ to approximately 150 γ per day, was sufficient to afford continued protection from sound-induced seizures, but with higher levels of pyridoxine, the seizures were both delayed and less severe.

These experiments appear to have demonstrated a correlation between the degree of pyridoxine deficiency and susceptibility to seizures. However, in view of the relatively large amounts of pyridoxine required to obtain a reduced sensitivity, it is possible that another dietary factor or factors in addition to pyridoxine may be of importance in the etiology of the seizures.

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THE AMINO ACID REQUIREMENTS OF LACTOBACILLUS ARABINOSUS 17-5

By D. MARK HEGSTED

(From the Division of Nutrition, Department of Biological Chemistry, Schools of Medicine and Public Health, Harvard University, Boston)

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Relatively little is known of the amino acid requirement of the lactic acid bacteria. Three species of heterofermentative lactic acid bacteria were studied by Wood, Geiger, and Werkman (1), and data are available for *Lactobacillus casei* (2), *Streptococcus lactis* R (3), and *Lactobacillus arabinosus* 17-5 (4, 5). All of these species require from eight to ten amino acids, but little is known of the specificity of these requirements.

The amino acid requirements of *Lactobacillus arabinosus* 17-5¹ have been investigated to extend our knowledge of the amino acid requirements of the lactic acid bacteria, and to study the possibility of using microbiological methods for the determination of amino acids.

EXPERIMENTAL

The basal medium used in these studies was similar to that of Snell and Wright (6), with the following changes. The casein hydrolysate, tryptophane, and cystine were replaced by an appropriate mixture of amino acids and 0.3 per cent ammonium sulfate. The glucose and sodium acetate concentrations were raised to 1.75 and 0.875 per cent respectively. Guanine hydrochloride, adenine sulfate, xanthine, and uracil were added to supply 4 parts per million and all of the vitamins including *p*-aminobenzoic acid but not biotin were raised to 0.4 part per million. It was thought desirable to add an ammonium salt to the medium, since Frantz (7) has shown that the amino acid requirements of the meningococcus are much simpler in the presence of added ammonium salts.

The ordinary procedures used in microbiological assays were followed (2, 6). The rate of growth was followed by titrating the lactic acid formed and also by determining turbidities at intervals in the Coleman spectrophotometer set at 580 m μ . The total volume of the medium used, however, was only 6 cc. per tube instead of the usual 10 cc. and titrations were made with 0.05 N sodium hydroxide after a suitable period of growth.

Results

A mixture of 0.5 mg. of each of nineteen amino acids per tube produced rapid growth. These were eliminated one by one from the mixture with

¹ Obtained from the American Type Culture Collection.

the results shown in Table I. Growth failed when *arginine*, *cystine*, *glutamic acid*, *isoleucine*, *leucine*, *methionine*, *phenylalanine*, *tryptophane*, *tyrosine*, or *valine* was omitted from the medium. Aspartic acid, alanine, lysine, and threonine appeared to be stimulatory, while histidine, serine, hydroxyproline, proline, and norleucine appeared to have little or no effect. The last three were later omitted from the amino acid mixture. When it became clear that the requirement for glutamic acid was high, the level of this amino acid was changed to 1.5 mg. per tube. This raised the maximum titration to about 14 cc.

Rather extensive studies to determine the minimum amino acid requirement showed that growth did not occur in a mixture of only the ten amino

TABLE I
Effect of Omitting Individual Amino Acids from Basal Medium*

Amino acid omitted	0.05 N acid per tube	Amino acid omitted	0.05 N acid per tube
	cc		cc
<i>l</i> (+)-Arginine monohydrochloride.	1 2	<i>l</i> (+)-Lysine monohydrochloride	6.55
<i>dl</i> -Aspartic acid	7.7	<i>dl</i> -Methionine	1.10
<i>dl</i> -Alanine	6 6	<i>dl</i> -Norleucine	8 80
<i>l</i> (-)-Cystine	1 2	<i>dl</i> -Phenylalanine	1 30
<i>l</i> (+)-Glutamic acid	0.8	<i>l</i> (-)-Proline	8 9
<i>l</i> (+)-Histidine monohydrochloride	8 65	<i>dl</i> -Serine	8.45
<i>l</i> (-)-Hydroxyproline	9.75	<i>dl</i> -Threonine	2.4
<i>dl</i> -Isoleucine	0 8	<i>l</i> (-)-Tryptophane	0 8
<i>l</i> (-)-Leucine	1.25	<i>l</i> (-)-Tyrosine	1.2
		<i>dl</i> -Valine	0.75

* All the amino acids were purchased from Merck and Company, Inc., Rahway, New Jersey.

acids shown to be essential for this organism. With a basal medium containing 0.5 mg. of each of these ten amino acids per tube further additions of amino acids were made with the following results. Aspartic acid allowed about three-quarters of maximum growth and by the further addition of threonine and lysine practically maximum growth was obtained. The addition of threonine only allowed a small amount of growth which, however, could not be subcultured. Threonine and lysine gave somewhat better growth but still considerably less than that obtained with aspartic acid alone. Lysine and aspartic acid gave no more growth than was obtained with aspartic acid alone. Regardless of the combination of amino acids added, growth failed when both threonine and aspartic acid were omitted from the medium.

Since Kuiken *et al.* (4) and Shankman (5) have recently reported that

threonine and lysine were essential amino acids for *Lactobacillus arabinosus* 17-5, these were reinvestigated. A 24 hour culture was washed three times with sterile saline according to the usual technique. After each suspension was made, 1 cc. was removed and diluted 10 times and seeded into a series of tubes containing lysine- or threonine-deficient media and graded amounts of these amino acids. The spectrophotometer readings at different times on the tubes inoculated with cells washed three times are shown in Table II. Although there appeared to be a graded response to various levels of threonine and lysine during the early part of the growth period, fairly good growth finally occurred in all of the tubes regardless of the level of threonine or lysine. Washing the cells actually appeared to stimulate growth rather than decrease it, as would have been expected had an essential nutrient

TABLE II

Spectrophotometer Readings (in Per Cent Transmission) Taken at Intervals at Various Levels of Threonine and Lysine

The basal medium contained all the amino acids except threonine or lysine.

Time <i>hrs.</i>	<i>DL</i> -Threonine added per tube				<i>L</i> -Lysine added per tube			
	0 γ	20 γ	40 γ	150 γ	0 γ	25 γ	50 γ	100 γ
15	90	88	87	78	78	73	72	68
20	86	76	72	39	31	36	35	19
38	35	25	21	11	10	10	10.5	10.5
44	20	18	14	9				
86	11	10	10	9				

been removed by washing. It is of interest to note that these studies were made after the amino acid composition had been changed as described above and much better growth was obtained on the deficient medium than is reported in Table I.

Considerable study was made of the effect of histidine, alanine, and serine in various combinations added to a medium containing the essential amino acids and aspartic acid, threonine and lysine, and the latter amino acids in various combinations. No clear cut evidence for a requirement of histidine, alanine, or serine has been obtained.

The quantitative requirement of all of the amino acids essential for this organism have been determined by adding graded amounts to a medium deficient only in the amino acid under investigation. Typical curves, such as are found in vitamin assays, were realized with cystine, tryptophane, phenylalanine, valine, isoleucine, and leucine. Certain inconsistencies were observed in the responses to methionine, arginine, tyrosine, and glutamic acid. These latter four have been investigated further and will be reported

in detail later. The quantitative requirements for the various essential amino acids are in the range reported by Shankman (5).

Preliminary studies of the use of the microbiological assay method for protein analysis are reported here for leucine, phenylalanine, and valine in casein and edestin, since they are suggestive of the results which can be obtained with the present method. The casein and edestin used for analysis were kindly supplied by Dr. H. B. Vickery. The hydrolysates were prepared by adding 0.5 gm. of the protein to 25 cc. of 6 N HCl and refluxing for 24 hours. The hydrolysates were then taken to near dryness repeatedly *in vacuo*, and finally neutralized, made to a given volume, pre-

TABLE III
Comparison of Activity of dl- and d-Valine

<i>dl</i> -Valine per tube	<i>d</i> -Valine per tube	0.05 N acid per tube
γ	γ	cc.
0		0.8
5		1.1
10		1.76
15		2.25
20		2.95
30		4.30
45		6.10
70		8.20
100		9.82
	10	0.4
	50	0.4
	100	0.4
	150	0.4
30	50	4.25
30	100	4.20
30	150	4.30

served by a layer of benzene, and kept in the ice box. These preparations were added in graded amounts to tubes containing all of the amino acids except the one under investigation and compared to a set of standard tubes after a suitable period of growth. Both spectrophotometer readings and acid titrations have been made. Titration usually gives more consistent results.

Valine—Typical values for *dl*-valine and those obtained with *d*-valine and graded levels of *d*-valine plus 30 γ of *dl*-valine are shown in Table III. It is apparent that *d*-valine is completely inactive and furthermore does not inhibit the response of *dl*-valine in the range tested. Thus only *l*(+)-valine is active. All results have been calculated on the basis that *dl*-valine is 50 per cent as active as the naturally occurring amino acid.

Analytical results for an assay of the valine content of casein and edestin are shown in Table IV. These data are not selected but represent one of a series of assays. As in all biological tests, considerable variation may be expected, but it would appear that the results of a reasonable number of tubes are as reliable as the vitamin assays in common use. It has been observed that in the tubes containing protein hydrolysates growth is initiated somewhat sooner and reaches its maximum before those containing only *dl*-valine. Since recovery of added valine is usually satisfactory, this effect may be similar to that observed by Feeney and Strong (8) with

TABLE IV
Valine Content of Edestin and Casein

	Protein added per tube	<i>l</i> -Val- ine* added	0.05 N acid titra- tion per tube	<i>l</i> -Valine†	Valine content	Aver- age	Valine‡ added as pro- tein	Added valine recovered		Aver- age
	mg.	γ	cc.	γ	per cent	per cent	γ	γ	per cent	per cent
Edestin	0.20		2.75	9.3	4.65					
	0.30		3.95	13.8	4.62					
	0.40		5.40	19.3	4.87	4.70				
	0.20	5	4.00	14.05			9.4	4.55	91	
	0.30	5	5.30	19.00			14.10	4.90	98	94.5
Casein	0.2		2.90	9.9	4.94					
	0.25		3.45	12.0	4.79					
	0.3		4.05	14.25	4.75					
	0.4		5.55	20.00	5.00	4.88				
	0.2	5	3.95	13.9			9.76	4.14	82.8	
	0.25	5	4.90	17.4			12.20	5.20	104.0	
	0.3	5	5.70	20.5			14.65	5.85	117.0	101

* Added as *dl*-valine.

† Determined from a standard curve with *dl*-valine.

‡ Calculated from the average in the previous column.

glutamine in pantothenic acid assays, when early growth was stimulated but the total amount of growth was unaffected.

Leucine—Leucine assays have been generally satisfactory with recovery values well within the range expected. Pure *d*-leucine has not yet been tested for activity and the sample of *dl*-leucine available appears to be somewhat less than half as active as the *l* form. This effect is being investigated.

Phenylalanine—The values for phenylalanine have been calculated on the basis that only the *l* form is active, although this has not been tested by actual assay.

Leucine, Valine, and Phenylalanine Content of Casein and Edestin—The

combined data from several assays for these amino acids are shown in Table V together with the per cent recoveries obtained by adding known amounts of the amino acid in question. Values from the literature are also given for comparison. No attempt has been made to select the data and these results probably represent the extreme variation that may be expected by the methods as presented.

TABLE V

Leucine, Phenylalanine, and Valine Content of Casein and Edestin As Determined by Microbiological Method

In the assays for phenylalanine and valine, the *dl* acid was used as a standard but the results are calculated on the basis that these are only half as active as the naturally occurring form. This has been proved by actual assay for valine but not for phenylalanine. *l*(+)-Leucine (Merck) was used as the standard for the leucine assays.

Amino acid	Casein				Edestin			
	Determined amino acid content	Recovery of added amino acid		Values from literature and bibliographic reference No.	Determined amino acid content	Recovery of added amino acid		Values from literature and bibliographic reference No.
		Range	Average			Range	Average	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Leucine	7.30	94-110	105	14.8 (9)	5.45	104-112	107	
				9.7 (10)				
	7.35			7.92 (11)	5.62			
				9.35 (12)				
	8.10	96-115	103	10.5 (13)	5.14			
	6.70							
Phenylalanine	3.70	74-110	92.5	5.8 (9)	4.02	82-112		3.09 (14)
				3.88 (10)				
				3.2 (13)	4.36			
Valine	5.20	89- 97	93	5.2 (9)	4.73	87-100	94	6.3 (15)
				7.93 (10)				
	4.87	83-117	101	7.20 (16)	4.70	93- 98	95.5	

DISCUSSION

Kuiken *et al.* (4) and Shankman (5) have reported that threonine and lysine are essential amino acids for *Lactobacillus arabinosus* 17-5. We have been unable to show that these are required. On the other hand, under our conditions, arginine, methionine, tyrosine, and phenylalanine were necessary. Kuiken *et al.* did not include the first three in their list of essential amino acids for this organism. Shankman reported that tyrosine and phenylalanine were required on one medium but was unable to show such a requirement when a different combination of amino acids was used in the basal medium.

It is possible, or even probable, that the nutritional requirements of *Lactobacillus arabinosus* cultivated in various laboratories may be different. However, close attention must also be paid to the constituents in the basal medium. In addition to the observation of Shankman concerning phenylalanine and tyrosine, Snell and Guirard (3) have shown that glycine, β -alanine, serine, and threonine are actually toxic to *Streptococcus lactis* in the presence of limiting amounts of alanine. Similar relationships for other amino acids have been reported by Gladstone (17), and Hutchings and Peterson (2) found that certain amino acids were inhibitory for *Lactobacillus casei*. If such relationships are general at the levels of amino acids used, the term "essential amino acid" may have significance only in relation to the composition of the basal medium.

The results reported in this paper together with other favorable reports on the microbiological determination of amino acids (4, 18) suggest that these limitations may not be too serious. It is apparent, however, that the critical range is quite limited in most assays. Values read from a position too high or too low on the standard curve often show considerable divergence from those obtained in the medial portion. Such limitations are also usually apparent in other microbiological assays.

SUMMARY

1. The single omission of arginine, cystine, glutamic acid, isoleucine, leucine, methionine, phenylalanine, tryptophane, tyrosine, or valine from an adequate medium containing nineteen amino acids prevents the growth of *Lactobacillus arabinosus*. However, a mixture of only these ten amino acids is not adequate for growth. The further addition of aspartic acid allows growth, but threonine, lysine, and aspartic acid are required in addition to the above ten amino acids for good growth.

2. The results of microbiological assays of casein and edestin for leucine, valine, and phenylalanine are reported.

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MEASUREMENT OF THIOUREA IN ULTRAFILTRATE OF SERUM

By THADDEUS S. DANOWSKI

(From the Department of Internal Medicine, Yale University
School of Medicine, New Haven)

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It has been suggested that thiourea, like urea, diffuses throughout all of the water in the body (1, 2).¹ If thiourea does permeate uniformly all water compartments and is not formed or destroyed in the body, it would fulfil the criteria of Winkler and Smith (3) as a measure of total body water. However, the ingestion or injection of large amounts of thiourea (6 to 10 gm.) produces malaise, nausea, and vomiting (1). Attempts to use less have been hindered by the lack of a procedure for the measurement of low concentrations of thiourea in serum. The use of protein-free filtrates, such as those obtained with the Somogyi procedure, trichloroacetic acid, or acetone, does not solve the problem. In the preparation of such filtrates the thiourea is diluted to very low concentrations, and substances are added which interfere with color reactions and titration procedures.

In this paper a method is described for the measurement of thiourea in the ultrafiltrate of serum in concentrations of 0 to 10 mg. per cent. It is based upon Nicholes' (4) use of the color reaction described by Grote (5) in which a specially prepared reagent reacts with the C=S portion of the thiourea molecule. Since thiourea is destroyed by direct contact with mercury, the Lavietes' capsule (6) cannot be used to produce the ultrafiltrate. Hence a closed system with nitrogen under pressure has been employed for the formation of ultrafiltrate of serum. With few adaptations the same method has been used for the measurement of thiourea in urine and in aqueous solutions.

Procedure for Analysis of Serum

Ultrafiltration of Serum—A volume of blood sufficient to yield 7 to 10 cc. of serum is withdrawn under oil. The serum is introduced by capillary pipette into cellophane cylinders. The cylinders are then incorporated into a system containing nitrogen under a pressure of 15 cm. of mercury and placed in stoppered tubes (Fig. 1). This pressure is maintained for 24 to 48 hours at temperatures of 6–10°. The refrigeration suppresses bacterial growth which makes the ultrafiltrate opalescent. The ultrafiltrate which forms on the outer surface of the cellophane is collected in the tube as 4 to 5 cc. of clear, colorless fluid. This fluid contains, in addition to the

¹ Lavietes, P. H., unpublished studies.

ultrafiltrable solutes of serum, the thiourea injected into the animal or added to the serum prior to ultrafiltration.

Grote's Reagent—0.5 gm. of sodium nitroferri-cyanide is dissolved in 10.0 cc. of distilled water at room temperature. To this solution 0.5 gm. of hydroxylamine hydrochloride is added, followed by 1.0 gm. of sodium bicarbonate. When the evolution of carbon dioxide ceases, 10 small drops of bromine are added. A second evolution of gas occurs. When agitation no longer produces effervescence, the mahogany-brown solution is filtered into a 25 cc. volumetric flask and made up to volume with distilled water. This Grote's reagent is diluted just before use by the addition of 4 parts of water.

Dilute Grote's reagent, even in the absence of thiourea, has a variable but significant amount of color, which absorbs light of the same wave-length

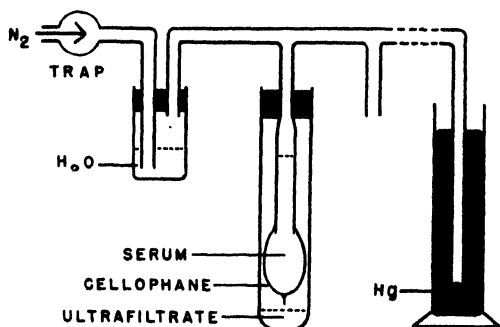


FIG. 1. Closed system of glass and cellophane used to form the ultrafiltrate of serum.

as that produced by its reaction with thiourea. The addition to this reagent of an ultrafiltrate known to be free from thiourea further increases the absorption of light. Studies were conducted with a common Grote's reagent and ultrafiltrates from seven patients and five dogs in fasting and non-fasting states. Provided that renal insufficiency, as manifested by an elevated non-protein nitrogen of the blood, was not present, the effect of the ultrafiltrate upon the amount of light absorbed is constant for as long as 49 days. However, since the color due to the Grote's reagent itself varies, the color of a mixture of Grote's reagent and ultrafiltrate was not constant in duplicate studies. This does not invalidate the method, since the factors responsible for this variation are present in both the standard and unknown solutions and affect the color of each to the same degree. It does necessitate, however, the construction of fresh standards for each group of determinations.

Standard Solutions—Thiourea² is recrystallized twice from water at a temperature no higher than 40°, and washed with alcohol and ether. It is then dried to constant weight in a desiccator and known solutions made which contain 3.0, 5.0, 6.0, and 8.0 mg. per cent of thiourea. Standards are then prepared with two or more of these solutions. To 9.0 cc. of distilled water in a colorimeter tube 1.0 cc. of ultrafiltrate of serum obtained prior to injection of thiourea is added, followed by 1.0 cc. of the known thiourea solution and 1.0 cc. of diluted Grote's reagent. This sequence must be followed, since the mixture of undiluted ultrafiltrate with Grote's reagent yields a less intense color. The tubes are then inverted several times and allowed to stand at room temperature. Within a few minutes a green, green-blue, or blue color develops with a maximum absorption of light of a wave-length of 560 to 610 μ (4). At the end of 60 minutes the light absorption of these standards is compared in an Evelyn colorimeter (aperture 10, Filter 565) with a control made without thiourea. The results are plotted semilogarithmically. A straight line is obtained even when four different concentrations of thiourea are used. The inclination of this line, however, varies, and an extension of the line does not pass invariably through the origin.

In the absence of renal insufficiency with azotemia, ultrafiltrate obtained from any serum can be used in the standards. Otherwise the ultrafiltrate obtained from the particular subject under study, prior to the administration of thiourea, must be used.

Unknown Solutions—The procedure with ultrafiltrate which contains an unknown amount of thiourea is identical with that followed in the preparation of standards, save that the ultrafiltrate is added to 10.0 rather than 9.0 cc. of water. Duplicate samples should give readings at 1 hour which differ by no more than a half division on the galvanometer scale, equivalent to 0.1 mg. per cent, or less. The concentration of thiourea in the unknown solution can then be read from the graph based upon standards prepared simultaneously with the unknown solutions.

Modification for Analysis of Urine

The procedure for the measurement of thiourea in urine differs in minor respects from that employed with ultrafiltrate. 2.0 or 4.0 cc. of urine are diluted with water to 100 cc. in a volumetric flask. 1.0 cc. of this diluted urine is mixed with 10.0 cc. of water and 1.0 cc. of Grote's reagent. Two or more standards are prepared from known solutions, water, and Grote's reagent. In contrast to the procedure with ultrafiltrate the sequence of addition is unimportant, and the green-blue color, once developed, is stable

² Obtained from the Eastman Kodak Company.

for at least 21 hours at room temperature. Hence it is not necessary that these solutions be read at exactly 1 hour. No urine blank is necessary, since dilution results in a zero value.

Characteristics of Color

A blue color with maximum absorption of light in the 560 to 610 μ band develops following the addition of Grote's reagent to a solution of thiourea.

Since the reagent is yellow in color, low concentrations of thiourea produce a green tint. This reaches maximum intensity in 10 to 20 minutes. In aqueous or saline solutions the color is stable for as long as 21 hours at room temperature. This is also true of solutions made with diluted urine containing thiourea.

In ultrafiltrate solutions, however, the color development is not as pronounced, and deterioration begins once it has reached maximum intensity. At room temperatures with *diluted* ultrafiltrate this deterioration is so slow that it is perceptible only in readings taken at 20 minute intervals. Deterioration can be accelerated by mixing Grote's reagent with undiluted ultrafiltrate. An increase in the temperature of the solution similarly hastens fading. Decrease of the temperature to 6–10° fixes the color for as long as 55 hours.

Identification was attempted of the factor or factors in the ultrafiltrate which make the color unstable. To aqueous solutions of thiourea sodium chloride, sodium bicarbonate, glucose, and urea were added in physiological amounts. None of these procedures conferred upon the aqueous solution the lability characteristic of the ultrafiltrate mixtures.

Repeated careful attempts to obtain a line graph of the standard solutions which could be duplicated invariably were unsuccessful. This was true even though the chemicals used in Grote's reagent were weighed to 0.5 mg. per cent. Use of either the original or freshly made Grote's reagent in successive determinations did not eliminate this variability. Exact duplication of time consumed in mixing the solutions produced no significant effect. Storage of the solution at refrigerator temperatures stabilized the color once it reached maximum intensity but did not yield identical curves.

At times an opalescent turbidity develops in the ultrafiltrate. This is the result of bacterial growth. It is undesirable, since it yields a false colorimetric reading. The frequency of this occurrence can be decreased by analysis of the ultrafiltrate soon after it is formed, or by storage at low temperatures. Attempts to prevent multiplication of the bacteria by the use of sodium cyanide or sodium sulfamerazine changed significantly the intensity of the color and the value of the control solution.

Sulfocyanate in concentrations of 4 to 10 mg. per cent does not interfere with the formation of color due to thiourea. The reverse, however, is not

true, if sulfocyanate is measured in serum. A false high concentration of color is obtained. This difficulty can be circumvented by analysis of the ultrafiltrate rather than of the serum. Hence, if use of both procedures is contemplated in the same animal, the sulfocyanate study should precede the thiourea, or the serum should be ultrafiltered.³

Recovery of Thiourea from Ultrafiltrate of Serum

8.0 cc. aliquots of 3, 5, and 8 mg. per cent solutions of thiourea were dried in duplicate at 37° during a period of 6 days. The residue was then made up to its original volume with serum and subjected to ultrafiltration in the manner described. The amount of thiourea in the ultrafiltrates was then compared with control solutions which had been subjected to an identical procedure, except that thiourea was added to the formed ultrafiltrate.

In six experiments of this type recovery of added thiourea was possible within the 0.1 mg. per cent limitation of the method.

SUMMARY

A colorimetric method is presented for the measurement of thiourea in concentrations of 0 to 10 mg. per cent in the ultrafiltrate of serum, in urine, and in aqueous solutions. A system is described for the production of an ultrafiltrate of serum by nitrogen under pressure with cellophane cylinders. Factors are discussed which affect the color produced by Grote's reagent in the presence of thiourea. Recovery of known solutions of thiourea added to serum and subjected to ultrafiltration was possible within 0.1 mg. per cent.

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USE OF THIOUREA AS A MEASURE OF CHANGE IN BODY WATER

By THADDEUS S. DANOWSKI

(From the Department of Internal Medicine, Yale University
School of Medicine, New Haven)

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During a series of experiments in this laboratory on the effect of water and food deprivation in dogs,¹ the possible use of thiourea as an index of total body water was investigated. Mongrel female dogs, 6 to 10 kilos in weight, were deprived entirely or partially of food or water, or both, for 6 to 29 days. During these intervals the animals invariably lost weight. The loss of water which contributed to the total decrease in body weight was calculated from metabolic balance studies. These results were then compared with changes in the apparent volume of distribution of thiourea.

Procedure

At the beginning and end of each period of deprivation the volume of distribution of thiourea was determined in the following manner. The animals were catheterized and weighed accurately. Blood was withdrawn for the preparation of a control solution and for the determination of the non-protein nitrogen. The dogs were then injected intravenously, by means of a calibrated 10 cc. syringe, with 5.0 cc. of a 10 per cent solution of recrystallized thiourea in normal saline. After 2 hours another sample of blood was obtained, the catheterization repeated, and the bladder washed twice with 10 cc. of water.

The concentration of thiourea in the ultrafiltrate of the serum and in the urine after 2 hours was determined by the method described in the preceding paper (1). The apparent volume of fluid through which the thiourea diffused was calculated from the usual formula

$$V = \frac{T_i - T_u}{T_s}$$

in which T refers to thiourea, and the subscripts i and u indicate, respectively, the amount in mg. injected and excreted; s refers to the concentration in serum in mg. per cent.

This procedure yielded an apparent volume of distribution at the start and end of each period. In the deprivation experiments the weight decline

¹ Danowski, T. S., Elkinton, J. R., and Winkler, A. W., unpublished studies.

was fractionated by means of metabolic studies into water and solids lost. During the convalescent period, however, metabolic studies were not attempted, and hence the total weight gain rather than the change in body water itself was employed as an approximate check upon thiourea as a measure of body water.

Results

Control Studies—Table I lists the results of ten thiourea distribution studies in four dogs. Six of these consist of two groups of three studies each in the same dogs, Nos. 36 and 37. The interval between studies varied from 3 to 9 days. During these periods the animals were fed adequately and offered water in sufficient amounts. Their weights, accurately determined at the time of the distribution studies, changed slightly from time to time. It is evident, however, that even these slight fluctuations in weight were reflected by a change in the apparent volume through which thiourea was distributed.

Studies of Change in Total Water—In Table II the results of twenty-three distribution studies in six dogs are recorded, with the successive changes in the apparent volume of distribution of thiourea. In eleven instances the fraction of the total weight loss due to a decrease in body water has been calculated from metabolic studies according to Lavietes' modification (2) of Newburgh's method (3) as used by Elkinton and Taffel (4) in dog studies. In each of these eleven pairs the algebraic sign of the change in the volume of water is the same, and a close quantitative correlation is evident. The results by the two methods do not differ from one another by more than 4 per cent of the animal's weight. In three instances they do not differ by more than 1 per cent.

Recovery of Thiourea—Four attempts were made to recover the thiourea injected (Table I). Analyses were made of the urine and the wash fluid from the bladder obtained after 2 hours, and of the urine collected during the next 3 days in a metabolism cage. The recovery of the injected thiourea varied from 50 to 82 per cent. Analysis of the fluid with which the floor of the metabolism cage was rinsed added but 5 to 8 mg. to the amount recovered. Analyses of pooled urine coincided with the results of examinations of separate daily specimens. Prolongation of the urine collection through the 9th day did not increase the percentage recovered. The stools were not analyzed for thiourea.

Toxicity of Thiourea—Nicolas and Lebduska (5) reported that 10 gm. of thiourea per kilo of body weight were fatal to dogs within 4 hours. Intravascular hemolysis occurred, and the animals died of respiratory failure following convulsions. Hemorrhages were present in the intestinal wall at autopsy. The intravenous solutions which they administered,

TABLE I
Apparent Volume of Distribution of Thiourea

Dog No.	Time	Weight	Volume of distribution	Δ weight	Δ water by Δ volume of distribution	3 days excretion
	<i>days</i>	<i>kg.</i>	<i>liters</i>	<i>kg.</i>	<i>liter</i>	<i>per cent of injection</i>
23	0	9.54	8.12			50
	3	10.00	8.85	+0.46	+0.73	75
32	0	6.70	5.12			73
	3	6.85	5.12	+0.15	0.00	82
36	0	7.68	7.10			
	9	7.00	6.51	-0.68	-0.59	
	15	6.74	6.21	-0.26	-0.30	
37	0	7.88	6.21			
	9	8.42	6.81	+0.54	+0.60	
	15	7.92	6.31	-0.50	-0.50	

TABLE II
Measurement of Change in Total Body Water

Dog No.	Food	H ₂ O	Duration	Weight	Δ weight	Thiourea volume of distribution	Body water as per cent of body weight	Δ water measured by*	
								Thiourea	Metabolic studies
			<i>days</i>	<i>kg.</i>	<i>kg.</i>	<i>liters</i>		<i>liters</i>	<i>liters</i>
23	+	+		9.94		7.52	76		
	0	0	7	8.12	-1.82	6.32	78	-1.20	-1.35
30	+	+		7.62		6.52	85		
	Glucose	0	8	6.34	-1.28	5.42	85	-1.10	-1.04
	+	+	26	7.54	+1.20	5.95	79	+0.53	
	0	0	10	6.00	-1.54	4.63	77	-1.32	-1.10
	+	+	17	7.06	+1.06	5.30	75	+0.67	
31	0	+	14	5.52	-1.54	4.17	75	-1.13	-0.99
	+	+		6.76		4.96	73		
	Glucose	0	7	5.40	-1.36	3.71	69	-1.25	-1.06
32	+	+		7.74		5.50	71		
	Fat	0	10	6.27	-1.47	4.08	65	-1.42	-1.35
	+	+	16	7.26	+0.99	4.78	66	+0.70	
	0	0	7	6.12	-1.14	4.16	68	-0.62	-0.88
	+	+	12	6.73	+0.61	4.74	70	+0.58	
	0	Saline	6	5.98	-0.75	4.16	70	-0.58	-0.57
	+	+	8	6.70	+0.72	5.12	76	+0.96	
35	+	+		7.30		5.58	76		
	0	0	10	5.86	-1.44	4.65	79	-0.93	-1.04
	+	+	17	6.96	+1.10	5.98	86	+1.33	
	Glucose	0	8	5.70	-1.26	4.80	84	-1.18	-1.07
37	+	+		7.92		6.31	80		
	Fish	0	29	6.72	-1.20	5.19	77	-1.12	-1.08

* The plus and minus signs indicate increases and decreases respectively.

however, were large in volume and not isotonic. It is likely that the death of the animals resulted from the injection of large amounts of fluid containing no salt, whose hemolytic effects on red blood cells would be identical with those of distilled water. Hence, it is not certain that even these huge amounts are harmful.

In these studies 5.0 cc. injections of a 10 per cent solution of thiourea in normal saline were used for a total dose of 0.500 gm. Occasional transient subdued behavior was observed following thiourea. Dog 36, however, invariably vomited after injection. Four animals died as a result of the deprivation of food and water. All had received thiourea within the preceding 24 hours. Postmortem examination revealed no gross changes in the tissues save for the presence of dehydration.

DISCUSSION

It is immediately apparent that thiourea seems to be distributed in some instances through a volume greater than that ordinarily assigned to the water in the body. This could be predicted from early observations not reported at this time. In those studies the volume of distribution was determined at intervals following a single injection. It was found that the apparent volume of distribution increased with time. A 2 hour period was selected as an easily reproducible interval for this comparative study when it was evident that, no matter what the interval used, a total volume of body water approximating 70 per cent could not be obtained consistently.

Though these distributions do not always fall within the range of the assumed physiological values, the control studies listed in Table I prove them to be quite constant. Slight variations in the weight of the dogs were reflected in a comparable change in the volume of distribution, both with respect to direction and order of magnitude. In eleven of the studies listed in Table II the change in body water was calculated both from metabolic balance studies and from changes in the apparent volume of distribution of thiourea. A close agreement is present in the results obtained by both procedures. Hence thiourea can be used to give evidence of a *change* in body water as an alternative method to the laborious metabolic studies.

It is possible that the high volumes of distribution are in some way related to the incomplete recovery of the injected material. Since thiourea is still present in the urine during the 3rd day, an active participation of thiourea in body metabolism does not seem likely. Yet there remains the possibility of a slow rate of metabolic activity with destruction of thiourea.

It is conceivable that thiourea enters some particular water compartment or is bound to some tissue in concentrations greater than its distribu-

tion throughout the rest of the body. Attempts to demonstrate this have been unsuccessful. The gradual release of such bound thiourea with subsequent excretion in the urine has not been detected. At no time has thiourea been found in the urine beyond the 3rd day following injection. It is possible that the thiourea, if bound, is released so slowly that it is lost during the process of dilution. Since the error of the method is 0.1 mg. per cent, dilution of the urine 1 part in 50 might well mask as much as 5 mg. per cent of thiourea. Attempts to circumvent this difficulty by analysis of the undiluted urine have been blocked by the variability in the value of the blank.

The question whether the tissue or compartment with the greater avidity for thiourea, if such there be, could become saturated by an initial injection of thiourea can be answered in part from the control studies in Table I. If saturation were produced by the initial injection, one would expect an increase in the second 2 hour level of thiourea, with a subsequent more complete recovery. This in turn ought to yield a volume of distribution approaching the theoretical body water. Thus far, however, only an increased recovery has been demonstrated without a marked change in the volume of distribution.

Use of the recovered rather than the injected amount of thiourea in the calculation of the volume of distribution does not yield consistently a percentage approximating 70 per cent of the body weight.

It would appear, hence, that a change in the apparent volume of distribution of thiourea can be used as an index of a *change* in the total water in the body of a dog. Occasional high volumes of distribution, however, preclude its use as a measure of the absolute amount of body water present.

SUMMARY

The volume of fluid through which injected thiourea appears to be distributed in dogs was determined in ten control studies and in twenty-three experiments during which the body weight and water changed. In eleven instances the change in the apparent volume of distribution was compared with the decrease in body water as determined by metabolic studies. Possible explanations for high volumes of distribution and for incomplete recoveries of thiourea are discussed.

1. Injected thiourea is distributed through a volume of fluid which either approximates or exceeds that assigned ordinarily to the total body water.

2. This volume is constant, provided the time allowed for diffusion is the same in duplicate studies.

3. A change in this volume is indicative of a change in the total volume of body water.

4. Measurement of a change in total water by studies of thiourea distribution agrees, within 4 per cent of the animal's body weight, with that obtained by metabolic calculation.

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LETTERS TO THE EDITORS

CRITICAL FACTORS IN THE RECOVERY OXYGEN CONSUMPTION OF RAT LIVER SLICES FOLLOWING ANOXIA IN VITRO*

Sirs:

Craig¹ recently reported that the oxygen consumption of liver slices following exposure to nitrogen *in vitro* was higher if the livers were taken from fed animals than if the rats had been fasted. Fasting also diminished the lactic acid output during the period of anoxia. The differences between fed and fasted animals may be due to the glycogen content of the liver, which has been suggested as a factor determining the rate of anaerobic glycolysis.²

The failure of liver slices from fasted animals to utilize glucose present in the medium during anaerobiosis suggests either irreversible damage to enzyme systems in the cells or the absence of some factor essential for glucose catabolism. There is evidence that potassium is essential for certain stages of carbohydrate metabolism.³ Glycogen is deposited in the liver together with potassium and water.⁴ *In vitro*, synthesis of glycogen from glucose by rat liver slices is most rapid in an "intracellular" medium containing high potassium concentration,⁵ and potassium is essential for carbohydrate synthesis in yeast.⁶ We have tested the influence of electrolyte composition of the medium on the recovery QO_2 following anoxia, using liver slices from fasted rats.

The experimental procedure was essentially that used by Craig. The Ringer-phosphate-glucose medium contained NaCl 0.12 M, KCl 0.0024 M, CaCl₂ 0.0017 M, MgCl₂ 0.0008 M, Na₂HPO₄ 0.017 M, NaH₂PO₄ 0.003 M, glucose 0.011 M. The "intracellular" medium contained K₂HPO₄ 0.054 M, KH₂PO₄ 0.013 M, MgHPO₄ 0.0085 M, MgSO₄ 0.01 M, glucose 0.011 M. The mean liver glycogen for all animals used, determined after a 24 hour fast, was 0.08 per cent.

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¹ Craig, F. N., *J. Biol. Chem.*, **150**, 209 (1943).

² Orr, J. W., and Stickland, L. H., *Biochem. J.*, **35**, 479 (1941).

³ Fenn, W. O., *Physiol. Rev.*, **20**, 377 (1940).

⁴ Fenn, W. O., *J. Biol. Chem.*, **128**, 297 (1939).

⁵ Hastings, A. B., *Harvey Lectures*, **36**, 91 (1940-41).

⁶ Pulver, R., and Verzár, F., *Helv. chim. acta*, **23**, 1087 (1940).

It is shown in the table that after 70 minutes exposure to nitrogen the subsequent QO_2 is greater in "intracellular" medium than in Ringer's solution, calculated either on an absolute or percentage basis. Lactic acid production during 1 hour's exposure to nitrogen was 1.6 mg. per gm. in Ringer's solution and 5.0 mg. per gm. in "intracellular" medium (mean values of duplicate determinations on three rats).

	Oxygen consumption, c.mm. per mg. initial dry weight per hr.	
	Ringer-phosphate-glucose	"Intracellular"-glucose
Initial QO_2	$8.68 \pm 0.18^*$	5.82 ± 0.32
QO_2 after 70 min. exposure to N_2	1.62 ± 0.14	3.37 ± 0.24
Per cent recovery of QO_2	18.6	57.9

* In each case, means and standard errors of the means for six rats.

It seems probable that in Ringer's solution the low QO_2 after anoxia of liver slices from fasted rats is due to the low glycogen content of the liver, and the inability of liver slices to utilize added glucose during the period of anoxia. The higher recovery QO_2 following anoxia and the higher lactic acid output during anaerobiosis, obtained in an "intracellular" medium, suggest that in the presence of larger amounts of potassium added glucose is made available for energy-yielding reactions essential to the cell during anaerobiosis.

Department of Physiology
Stanford University
California

FREDERICK A. FUHRMAN
 J. M. CRISMON

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THE EFFECT OF STAGE OF GROWTH ON THE CHEMISTRY OF THE GRASSES

By GEORGE O. KOHLER

(From the Cerophyl Laboratories, Inc., Kansas City, Missouri)

(Received for publication, November 12, 1943)

The effect of age on the chemical composition of various grasses has been the subject of much research. As the result it is common knowledge that "young" grasses are richer in protein, soluble carbohydrates, carotene, vitamin C, thiamine, and riboflavin than are older grasses (1-15). The cellulose and other structural constituents increase as the plant ages. Hence, young grasses or grasses kept in a vegetative condition by cutting or grazing are nutritionally superior to older grasses.

In addition to this type of experiment (1-15) in which "young" grass usually refers to grass at least 3 to 6 weeks of age, a number of studies have been carried out on sprouting seeds. It has been found that chlorophyll, xanthophyll, and carotene (16-18), as well as the water-soluble vitamins (19-22), increase during and subsequent to germination. In the case of some of the constituents, the maximum points between germination and the old plant have been observed. Such maxima must exist for all of the vitamins as well as protein, chlorophyll, and many other constituents.

The work described in the present paper was carried out to determine when the various constituents reach their maximum concentrations, and to attempt to correlate the analytical data with the physiological stage of growth and the rate of growth of the plant.

EXPERIMENTAL

Oats were selected as a typical example of a cereal grass. The variety Kanota was used, since previous experience indicated that it is well adapted to the environmental conditions likely to be encountered in eastern Kansas. The experiment was carried out on a carefully selected uniform field near Lawrence, Kansas, the soil being a chernozem type with a high base exchange capacity and organic matter content, but a fairly low per cent base saturation. The seeds were sown with standard farm equipment at a rate of $4\frac{1}{2}$ bushels per acre on the 28th of April.

After the plants had grown to a height of 6 to 8 inches (May 22, 1941), two sets of samples were taken every 3 days until grain heads appeared. The first set of samples was taken as follows:

Twenty-five plants were hand-picked for physical examination, dissection, and measurements. The tiller culms were separated from the seed

culms, and the roots were removed at the crown. They were dried at 80° and ground, and protein analyses were run on these two fractions of the plant. The second set of samples was obtained by the use of a commercial harvester of the type ordinarily used in the production of dehydrated grass or alfalfa. It cut the grass 1 to 1½ inches above the ground. A single swath 5 feet by 100 to 400 feet was cut across the field for a sample.¹ Samples were cut so as to eliminate border effect. The grass was then transported by truck to the dehydrating plant where it was immediately dried under commercial conditions in an Ardrier. After dehydration, the samples were ground to pass a 32 mesh screen in a hammer mill. In this way, 50 to 100 pound samples were obtained which were placed in cold storage (-12.2°) to await analysis. Earlier work had shown this method of storage to be suitable for preservation of the factors to be determined. Previous to analysis, the 100 pound bags were sampled with a grain sampler (six stabs per bag). The samples were then warmed to room temperature in a desiccator and were rolled and quartered according to standard technique.

Records of average temperature and humidity were kept and rainfall was recorded. These are given in Table I.

Methods of Analysis

Biotin (23), *Lactobacillus casei* growth factor² (norit eluate factor) (24), and pantothenic acid (25) were all tested by the microbiological method with *Lactobacillus casei* ϵ as test organism. A similar procedure with *Lactobacillus arabinosus* (26) was used for nicotinic acid. Riboflavin was determined both fluorometrically (27), with a Lumetron photoelectric colorimeter, and by the microbiological procedure (28). Satisfactory checks were obtained. All of the procedures for microbiological assay were modified by addition to the basal media of synthetic vitamins and a concentrate of the eluate factor prepared from grass. Thiamine was determined by the thiochrome method by use of an internal standard. Carotene determinations were run by both partition (29) and chromatographic methods (30). An Evelyn photoelectric colorimeter was used to determine the carotene content of the purified extracts. Chlorophyll was determined with a Beckman photoelectric spectrophotometer. The optical density

¹ The May 9 sample was collected by hand, since the rain (see Table I) had softened the field and made it impossible to use the harvester.

² The *Lactobacillus casei* factor was determined with a concentrate containing 7.7 per cent folic acid as standard. This material was generously supplied by Dr. E. E. Snell. Since a number of different compounds have growth-promoting activity for *Lactobacillus casei*, the values given should be considered relative rather than absolute.

of ether solutions of the fat solvent-soluble constituents was determined at wave-lengths of 6600 and 6425 Å. Chlorophyll *a* and *b* concentrations were calculated from the absorption coefficients of the pure compounds as determined by Comar and Zscheile (31). Ascorbic acid was determined by titration with 2,6-dichlorophenol indophenol (32). Grit analyses were run according to the procedure of the Association of Official Agricultural Chemists with carbon tetrachloride instead of chloroform for flotation of the grass from the soil which is entrapped at the base of the leaf blade and

TABLE I
Environmental Data during Experimental Period

Date	Average temperature	Rainfall	Relative humidity
	°C.	inches	per cent
May 22	20.0	0.75	75
" 23	15.0	0.00	55
" 24	17.7	0.00	42
" 25	22.7	0.00	54
" 26	25.0	Trace	57
" 27	25.5	0.00	61
" 28	26.1	0.00	66
" 29	25.0	0.00	64
" 30	24.4	0.25	80
" 31	20.5	0.39	91
June 1	22.7	0.63	77
" 2	21.6	Trace	77
" 3	18.3	"	89
" 4	20.6	0.00	63
" 5	23.3	0.00	59
" 6	20.5	Trace	88
" 7	22.2	0.00	68
" 8	20.5	2.00	
" 9	21.1	1.13	82
" 10	20.5	Trace	80
" 11	19.4	"	73
" 12	17.2	0.00	68

under the sheath. All results were calculated on a moisture-free, grit-free basis.

Results

Data on the growth rates and tillering are given in Table II. It will be noted that the number of tillers per plant shows only a slight tendency to increase with age. In view of the fact that the total weight of the tillers formed after the first sample is relatively small, it is improbable that the analytical results on the dehydrated samples were affected appreciably by

this factor. Fig. 1 shows that growth (as dry weight and height) of the seed culms proceeded as a logarithmic function of time. Therefore, both weight and height changes may be expressed mathematically by the general growth equation, $X = Ae^{kt}$ (33). The increase in dry weight of the seed culms may be calculated by the formula $W = e^{0.091t}$, where e is the base of

TABLE II

Results of Physical Measurements and Observations on Hand-Picked Sample

Days of growth (from planting)	Seed culms				Tiller culms				Total plant, average weight
	Average height	Average weight	Joints	Heads out	Average No. per seed culm	Average height	Average weight	Joints	
	inches	gm.	per cent of total culms	per cent		inches	gm.	per cent	
24	9.8	0.0868	0	0	1.52	6.3	0.0307	0	0.1286
27	10.8	0.0987	0	0	1.76	7.1	0.0334	0	0.1552
30	12.6	0.1900	32	0	1.88	8.2	0.0669	5.5	0.3348
33	14.1	0.1755	12	0	1.96	10.3	0.0621	0	0.2993
36	15.6	0.3170	52	0	2.20	11.0	0.2057	24.0	0.546
39	18.4	0.3032	52	3	1.32	11.5	0.0932	11.1	0.4265
42	19.7	0.4562	72	16	2.44	10.6	0.1642	28.1	0.8780
45	24.7	0.5795	84	28	1.00	10.2	0.1718	32.0	0.7520

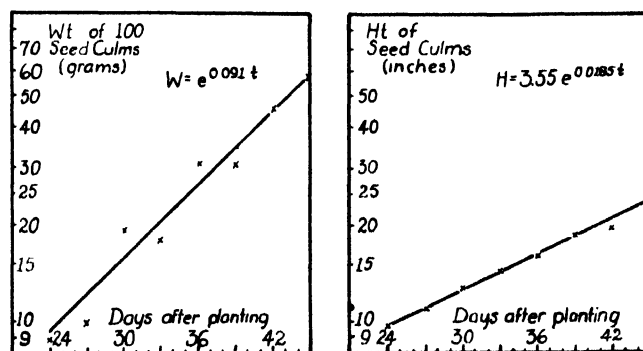


FIG. 1. Growth of the seed culms of oat grass plotted on semilogarithmic paper

natural logarithms and t is the time (days) from planting. 0.091×100 gives the percentage increase in weight per day. Similarly, the changes in height of the seed culms may be calculated from the formula $H = 3.55e^{0.0185t}$. The growth of the tiller culms does not follow this type of formulation, possibly because of the fact that these figures represent the average of a number of culms of physiologically different ages.

The percentage of jointed culms is also given in Table II. The jointing stage refers here to the stage at which the internodal tissue begins to elongate to form the stem of the plant. The work of Bonnett (34) has shown that previous to this stage in the individual culm "the growing point remains short, the leaf initials differentiate, leaves grow, and tiller buds develop in the axils of the leaves at the base of the stem. During the second stage, the internodes of the stem elongate, and the branches, spikelets, and flower parts differentiate and develop." The protein contents of the seed and tiller culms are shown in Fig. 2. A comparison of Table II and Fig. 2 shows that the protein content of the culms is a function of physiological age. The rapid decline of protein corresponds to the rapid formation of joints in both seed and tiller culms. The lower protein values of seed and

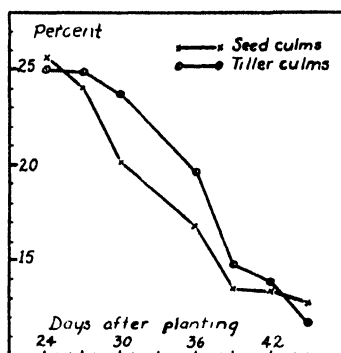


FIG. 2. Effect of age on protein content of the seed and tiller culms from the same plants (hand-picked samples).

tiller culms as compared with those obtained on the dehydrated samples may be attributed to the fact that the former included the entire culm above the root (Fig. 2). The dehydrated samples were obtained from grass cut 1½ inches above the ground (Fig. 3).

By the 39th day, the first grain heads appeared, although even by the 42nd day only 16 per cent of the seed culms was headed out.

The analytical results on the dehydrated grass samples are shown in Fig. 3. The factors may be divided into four groups as follows: (1) those which showed maximum concentrations at or just previous to the jointing stage (this group includes crude fat (petroleum ether extract), protein, chlorophyll, carotene, riboflavin, thiamine, ascorbic acid, and the *Lactobacillus casei* growth factor (norit eluate factor)); (2) those which show their maxima considerably earlier than the jointing stage (nicotinic acid is the only representative of this group which has been found); (3) factors which

reach their maximum concentrations between the jointing and the heading stages (these include biotin and pantothenic acid); and (4) structural constituents which increase in concentration up to the heading stage, represented here by crude fiber.

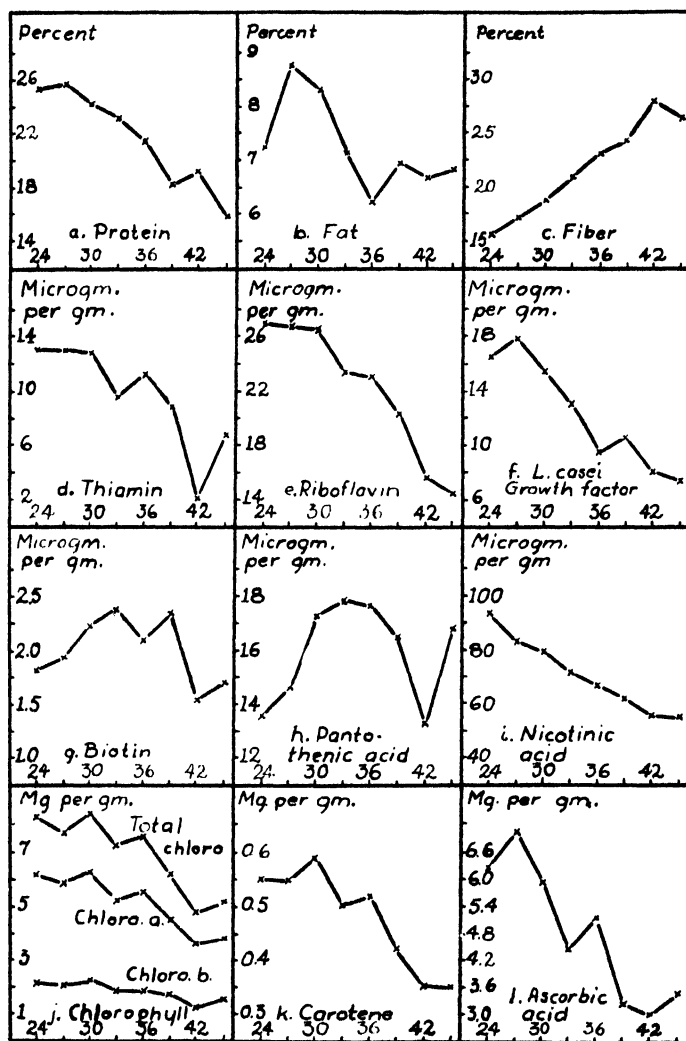


FIG. 3. Effect of age on the composition of the oat plant. The samples were harvested with a commercial harvester (about 1½ inches above the ground), and dehydrated by a flash process. The abscissae are in terms of days after planting.

stituents which increase in concentration up to the heading stage, represented here by crude fiber.

DISCUSSION

It is fortunate that the temperature conditions remained so nearly constant throughout the experiment, since, of course, the value of K in the growth equation is dependent on temperature in so far as plants are concerned. In view of the developmental changes which occur at the jointing stage, a change in slope of the logarithmic growth curve might be expected at this point. Neither these data nor those presented by Brody (35) contain enough points previous to the jointing stage to establish whether there is a break in the curve or not. Further work is in progress in this laboratory on this point.

It is seen from the data that most of the vitamins and the protein, crude fat, and chlorophyll reach a peak in concentration at or near the jointing stage. These results are in accord with those of Schnabel (36), who found that the "nutritive value" of grass was optimum at this point.

At this stage, the plant is at the peak of its vegetative development. Up to the jointing stage, the aerial part of the plant is made up entirely of leaf blades and sheaths. As the seedling grows and the leaves unfold, more and more surface is exposed to sunlight, and, hence, can partake in photosynthetic activity. Thus, factors which presumably play important metabolic and photosynthetic rôles might be expected to increase up to this point. Elongation of the internodes results in the formation of a stem containing a high percentage of structural constituents. Thus, the relative amount of actively metabolizing tissues becomes smaller.

That the pantothenic acid and biotin values reached maxima considerably later may indicate that they function in enzyme systems not directly associated with photosynthesis. Their maxima coincide with the rapid deposition of cellulose and other structural constituents as well as with the rapid development of the branches, spikelets, and flower parts (34). It will be noted that the last two points on the pantothenic acid and thiamine curves are considerably off the smooth curve which may be drawn through the other six points. This may be related to the heavy rainfall mentioned above. Fiber, protein, ascorbic acid, biotin, and chlorophyll also show irregularities at this point, although of a lesser degree. The other constituents determined did not appear to be affected appreciably by the excessive moisture.

No suggestion is offered in explanation of the early nicotinic acid peak.

It is apparent from the above data that the physiological stage of growth is of prime importance in evaluation of the effects of all types of environment on the quality of the grasses. It is difficult to interpret much of the published work on grasses because this factor has not been taken into account. In studies on fertilization, varieties, etc., the cuttings should be

carefully controlled on the basis of physiological age rather than chronological age.

SUMMARY

1. The immature oat plant has been studied at various stages of development. Protein, fat (petroleum ether extract), fiber, chlorophyll, carotene, riboflavin, ascorbic acid, thiamine, biotin, pantothenic acid, *Lactobacillus casei* factor (norit eluate factor), and nicotinic acid have been determined.

2. The concentrations (per gm. basis) of these constituents have been correlated with the physiological stage of growth of the plant.

3. Most of the factors showed a maximum concentration at or about the jointing stage. Pantothenic acid and biotin showed later maxima, while nicotinic acid was present in maximum concentration at an earlier stage. The concentration of crude fiber continued to increase throughout the experiment.

4. Growth rate of the seed culms proceeded according to the general equation, $W = Ae^{kt}$.

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SOME BIOLOGICAL EFFECTS PRODUCED BY BENZIMIDAZOLE AND THEIR REVERSAL BY PURINES

By D. W. WOOLLEY

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

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The production of thiamine deficiency in animals and many microorganisms by the administration of pyrithiamine, the pyridine analogue of thiamine (1), has prompted us to attempt the synthesis of analogues of other biologically important compounds by application of the same type of structural change as that involved in passing from thiamine to pyrithiamine. Thus the formation of the compound derived from biotin by the substitution of $-\text{CH}:\text{CH}-$ for the sulfur atom seemed advisable. While we were engaged in the synthesis of this analogue, an abstract of a paper appeared (2) which described the pharmacological properties of benzimidazole. The structural similarity of this compound to the biotin analogue in which we were interested was apparent. Furthermore, the similarity of the symptoms observed in animals receiving benzimidazole to those seen in biotin deficiency suggested that the action of benzimidazole might be related to its structural similarity to biotin. In order to determine whether benzimidazole owed its biological potency to competition with biotin, the production of biotin deficiency was attempted in several microorganisms by addition of benzimidazole. However, while the growth of these microorganisms was shown to be inhibited by benzimidazole and certain of its derivatives, this effect was not overcome by biotin. The close structural similarity of benzimidazole to purine then suggested itself, and tests soon demonstrated that the effect on growth was overcome by adenine. Trials with other purines showed that the inhibition of growth was overcome only by the aminopurines, adenine and guanine. The other purines which were tested were ineffective.

Certain structural features were necessary in the benzimidazole in order for it to have growth-inhibiting powers for yeast. Thus, when the hydrogen atom of position 2 was replaced by a side chain or by hydroxyl, activity was destroyed or greatly diminished. Since the substitution of an amino group in position 4 of benzimidazole would result in a compound more analogous to adenine, and the substitution of an amino group in position 5 would yield one more analogous to guanine, it was hoped that such amino compounds would be much more active than unsubstituted benzimidazole. However, 4-aminobenzimidazole and 4-nitrobenzimidazole had approxi-

mately the same potency as benzimidazole when judged on a molecular basis. 5-Aminobenzimidazole was somewhat less than half as active.

The syntheses of the two aminobenzimidazoles and the position of the nitro group in nitrobenzimidazole have not been known previously. 5-Aminobenzimidazole was synthesized by the condensation of formic acid with 1,2,4-triaminobenzene. Bamberger and Berl  (3) showed that nitration of benzimidazole introduced a nitro group at an unknown point in the benzene ring. Since only two isomeric compounds are possible, reduction of the nitrobenzimidazole and comparison of the resulting aminobenzimidazole with 5-aminobenzimidazole would establish the structure of the nitro compound. Bamberger and Berl  were unable to prepare the pure amine by reduction with tin and hydrochloric acid, but we have found that the preparation was easy when ferrous sulfate and ammonia were used. The aminobenzimidazole thus prepared differed in melting point and in biological activity from 5-aminobenzimidazole and hence was 4-aminobenzimidazole.

Along with sulfanilamide, benzimidazole is the only inhibitor related structurally to the vitamins and acting competitively with them which has been found effective against organisms that are able to synthesize the vitamin to which the inhibitor is related. In the case of pyridine-3-sulfonic acid (4) (related to nicotinic acid), thiopanic acid (5, 6) (related to pantothenic acid), and pyrithiamine (1) (related to thiamine), the inhibitor was effective only against organisms which required the related vitamin preformed in the medium. However, just as sulfanilamide was not limited in its inhibitory action to organisms for which *p*-aminobenzoic acid was required as a growth factor, so also benzimidazole was not limited in its action to those organisms unable to synthesize aminopurines.

The reversal of benzimidazole action on microorganisms by purines suggested the hypothesis that benzimidazole owed its anesthetic and other pharmacological properties to its competition in the animal with the aminopurines. However, it was not possible to prevent anesthesia in animals injected with benzimidazole by the administration of adenine. Some reduction in the length of the period of anesthesia was produced by adenine, but the results were not as striking as in the case of the growth of microorganisms. Nevertheless, it is still possible that the pharmacological action of this new drug is related to the purines.

EXPERIMENTAL

Sources of Benzimidazoles—Benzimidazole itself was obtained from commercial sources. 2-Methylbenzimidazole and glucobenzimidazole were synthesized according to the directions of Moore and Link (7). 2-Hydroxybenzimidazole was prepared by the action of phosgene on an ether solution

of *o*-phenylenediamine. Nitrobenzimidazole was prepared according to the method of Bamberger and Berl  (3), and 2-hydroxyimidazole according to that of Marekwald (8).

5-Aminobenzimidazole—25 gm. of 1,2,4-triaminobenzene trihydrochloride, 4.6 gm. of formic acid, and 40 cc. of water were placed in a 100 cc. flask which carried an air condenser. The flask was heated in an oil bath until the contents were homogeneous. 5 cc. of phosphoric acid were added, and the temperature was held at 135° for 3 hours. The black, semisolid product was dissolved in 400 cc. of water. The solution was treated with excess concentrated ammonium hydroxide, and evaporated on a steam bath to half its volume. 12 gm. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ were dissolved in 200 cc. of water, and just enough ammonium hydroxide was added to give a clear solution, and this solution was added to that of the reaction product. The precipitate which formed was filtered off, washed with water, suspended in dilute HCl, and decomposed with H_2S . The filtrate from CuS was treated with excess ammonium hydroxide and concentrated under reduced pressure to about 100 cc. 6 gm. of dark purple crystals formed as the solution stood in the cold. The product was purified by recrystallizing it twice from water with the addition of much norit, and was obtained as 1.1 gm. of slightly pink crystals; m.p.¹ 105–106°. The compound turned slightly purple when it was dried at 60°.

$\text{C}_7\text{H}_7\text{N}_3 \cdot 2\text{H}_2\text{O}$. Calculated. C 49.7, H 6.5, N 24.8
Found. " 50.4, " 5.8, " 24.5

4-Aminobenzimidazole—10 gm. of nitrobenzimidazole (3) were dissolved in 300 cc. of water and 50 cc. of N HCl, and the solution was treated with 120 gm. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 200 cc. of water. The solution was stirred and treated with an excess of ammonium hydroxide. The mixture was heated to boiling, filtered, and the precipitate was washed with hot water. The yellow filtrate was concentrated under reduced pressure to about 60 cc. and allowed to stand in the cold. The crystals were filtered off, washed, and recrystallized from water; yield 5 gm.; m.p. 104–105°; mixed melting point with 5-aminobenzimidazole 100–102°. Like the isomeric 5-aminobenzimidazole, this compound darkened when dried at 60°, and thus in this respect differed markedly from the analogous 6-aminopurine.

$\text{C}_7\text{H}_7\text{N}_3 \cdot 2\text{H}_2\text{O}$. Calculated. C 49.7, H 6.5, N 24.8
Found. " 49.2, " 5.8, " 24.6

Method of Microbiological Tests—In order to test the effect of benzimidazole on the growth of yeast, suitable graded amounts of the compound were added to flasks containing 10 cc. of the medium described below.

¹ All melting points were uncorrected.

The flasks were then sterilized by autoclaving for 15 minutes at 15 pounds pressure, cooled, and inoculated with 1 drop per flask of a diluted inoculum, prepared as previously described (9). The flasks were then incubated (at 30° if *Saccharomyces cerevisiae* was used, and 37° if *Endomyces vernalis* was employed) for 20 hours. The turbidity of the contents of each flask was next determined photometrically as described earlier (9), and from the values so obtained a curve was constructed relating the amount of growth to the quantity of compound added. From this curve, the quantity necessary to produce half maximal inhibition of growth was ascertained. The medium had the following composition: glucose 20 gm., $(\text{NH}_4)_2\text{SO}_4$ 3 gm., KH_2PO_4 2 gm., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25 gm., $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.25 gm., ZnSO_4 1 mg., MnCl_2 1 mg., FeCl_3 0.5 mg., $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.1 mg., KI 0.1 mg., *l*-asparagine 100 mg., inositol 5 mg., calcium pantothenate 0.5 mg., pyridoxine 0.02 mg., biotin² 0.01 mg., thiamine 0.1 mg., and water to make 1 liter.

When bacteria were used as test organisms, the procedure was the same as that for yeast, except that the media were of different composition. In addition, for those bacteria which produced acid, the results of the turbidimetric determinations were checked by titration of the acid produced. The titrimetric results agreed well with those obtained by turbidimetry. The medium for *Escherichia coli* was composed of glucose 10 gm., acid hydrolysate of casein (9) 2 gm., K_2HPO_4 4 gm., KH_2PO_4 2 gm., NaCl 3 gm., Salts B (10) 4 cc., in 1 liter of water adjusted to pH 7.

The medium for *Streptococcus lactis* R was composed of glucose 10 gm., acid hydrolysate of casein 5 gm., sodium acetate 10 gm., tryptophane 50 mg., cystine 100 mg., thiamine 100 γ , pyridoxine 100 γ , calcium pantothenate 100 γ , riboflavin 200 γ , nicotinic acid 100 γ , biotin 0.2 γ , inorganic Salts A and B (10) 2.5 cc. each, folic acid concentrate (11) 50 mg., and distilled water to make 1 liter.

Effect of Benzimidazole on Growth of Saccharomyces cerevisiae—Growth of this yeast was completely prevented by 600 γ or more of benzimidazole per cc. Half maximal effect was produced by 300 γ per cc. Some representative data are shown in Table I.

Effect of Other Benzimidazoles on Growth of Saccharomyces cerevisiae—The relative potency of various benzimidazoles as measured by the amount necessary to cause half maximal inhibition of growth was shown by the data in Table II. It can be seen that substitution in position 2 reduced or eliminated activity against this yeast. Substitution of nitro or amino groups in the benzene part of the molecule did not greatly alter activity.

Stimulation of Yeast by Benzimidazoles—Stimulation of the growth of

² We wish to thank Dr. J. C. Keresztesy, of Merck and Company, Inc., for gifts of crystalline biotin.

Saccharomyces cerevisiae was produced by subinhibitory amounts of benzimidazole. This stimulation was slight but readily reproducible (Table I). Glucobenzimidazole (1 mg. per cc.) and 2-hydroxybenzimidazole (0.5 mg.

TABLE I
Inhibitory Effects of Graded Amounts of Benzimidazole on Growth of Saccharomyces cerevisiae and Their Reversal by Adenine

Benzimidazole	Adenine sulfate	Turbidimeter reading*
γ per cc.	γ per cc.	
0	0	69
100	0	69
200	0	66
300	0	84
500	0	96
600	0	99
1000	0	100
600	1000	69
600	600	83
600	300	93
1000	2000	80

* Turbidimeter readings were expressed as per cent of the incident light transmitted by the cultures, and hence were inversely proportional to the amount of growth.

TABLE II
Relative Activity of Various Benzimidazoles in Causing Inhibition of Growth of Saccharomyces cerevisiae

Compound	Amount necessary to cause half maximal inhibition
	γ per cc.
Benzimidazole.....	300
2-Methylbenzimidazole...	500*
Glucobenzimidazole	No effect with 5000
2-Hydroxybenzimidazole...	" " " 500
2-Hydroxyimidazole.....	" " " 500
4-Nitrobenzimidazole.....	450
4-Aminobenzimidazole....	400
5-Aminobenzimidazole....	1200

* Complete inhibition of growth was not observed even with 2000 γ per cc.

per cc.) produced more stimulation than did benzimidazole. With these compounds, the stimulation corresponded to a decrease over the control flasks of 8 in the turbidimeter reading.

Reversal of Effect of Benzimidazole with Purines—The inhibitory effect of benzimidazole was overcome completely by sufficient amounts of adenine

or guanine. Representative data obtained with adenine are shown in Table I. It will be noted that the ratio of benzimidazole to adenine required for reversal was about the same with high as with low doses of benzimidazole. Wide ranges of concentration could not be used to investigate this phenomenon more fully because of the low solubility of both substances.

The relative effectiveness of several purines and derivatives in overcoming the effect of benzimidazole is shown in Table III. Guanine was the most active substance tested, but its insolubility limited its use. It will be seen that yeast adenylic acid and muscle adenylic acid, as well as the hydroxypurines derived from the active aminopurines, were ineffective. Uracil was inactive, although for *Streptococcus lactis* R it was the most active

TABLE III
Relative Activity of Various Purines in Overcoming Effect of Benzimidazole on Saccharomyces cerevisiae

Compound	Amount required for half maximal reversal*
	γ per cc.
Adenine sulfate.....	600
Guanine hydrochloride.	100
Hypoxanthine.....	No effect with 500
Xanthine.....	" " " 300
Yeast adenylic acid....	" " " 500
Muscle " "	Slight effect with 500†
Uracil.....	No effect with 1000

* These quantities were determined in the presence of 600 γ of benzimidazole per cc.

† 500 γ of muscle adenylic acid per cc. caused 0.1 maximal reversal; smaller amounts gave no effect.

substance (see below). Several vitamins did not reverse the effect of benzimidazole. Thus thiamine (10 γ per cc.), biotin (10 γ per cc.), crystalline folic acid³ (0.01 γ per cc.), and *p*-aminobenzoic acid (100 γ per cc.) were without effect.

Inhibition of Other Microorganisms with Benzimidazole—Several species of bacteria and yeasts were prevented from growing by approximately the same concentration of benzimidazole as was found effective against *Saccharomyces cerevisiae*. Data to illustrate this point are shown in Table IV. In all instances except with *Streptococcus lactis* R, the addition of adenine reversed the effect. For this organism, the concentrations of adenine which could be expected to prove effective were themselves inhibitory.

³ We wish to thank Dr. A. D. Emmett, of Parke, Davis and Company, for a gift of crystalline folic acid.

However, in further contrast with the results with yeast, uracil was quite active in reversing the effect of benzimidazole on *Streptococcus lactis* R. 10 γ of uracil per cc. produced half maximal reversal of the action of 1.5 mg. of benzimidazole per cc. Nevertheless, it was not possible to bring about complete reversal with any amount of uracil up to 1 mg. per cc.

Attempts to Overcome Anesthesia Produced in Mice by Benzimidazole—The anesthesia produced in mice was not prevented by adenine. Thus fifteen mice which were given 10 mg. each of benzimidazole and 15 mg. each of adenine sulfate intraperitoneally were anesthetized for approximately 1 hour, while fifteen control mice which received only the benzimidazole were anesthetized for approximately 1.5 hours. Three of six mice, which were given 30 mg. of adenine sulfate intraperitoneally a few minutes after they had been anesthetized with 10 mg. of benzimidazole, revived partially in a few minutes, but they soon began to fail and died within a few hours,

TABLE IV
Relative Effectiveness of Benzimidazole against Microorganisms

Organism	Amount necessary for half maximal inhibition
	γ per cc.
<i>Saccharomyces cerevisiae</i>	300
<i>Endomyces vernalis</i>	300
<i>Escherichia coli</i>	370
<i>Streptococcus lactis</i> R.....	725

whereas the controls survived. It was concluded that there was no proof that adenine antagonized the anesthetic action of benzimidazole in mice.

SUMMARY

Benzimidazole was shown to inhibit the growth of several yeasts and bacteria. This action was completely negated by aminopurines. The structural similarity of purines to benzimidazole was noted. Several benzimidazoles were synthesized and examined for growth-inhibiting action, and it was shown that no substitution could be made in position 2 if full activity was to be maintained. Only adenine and guanine were effective in reversing the action of benzimidazole. Of these, guanine was the more active. Several other purines were not effective. Anesthesia produced in mice by benzimidazole was not prevented by the simultaneous administration of adenine.

5-Aminobenzimidazole and 4-aminobenzimidazole were synthesized, and the structure of nitrobenzimidazole was shown to be 4-nitrobenzimidazole.

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NOTES ON THE MANOMETRIC HYPOBROMITE ESTIMATION OF UREA

By RAYMOND F. HOLDEN, JR.

(From the Department of Internal Medicine, Washington University
School of Medicine, St. Louis)

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During the course of work involving frequent determination of plasma urea by the convenient manometric method of Van Slyke and Kugel (1),

TABLE I

Comparison of Urea Nitrogen by Urease Method of Van Slyke (2) on Tungstic Acid Filtrates and by Hypobromite Method of Van Slyke and Kugel (1) on Ferric Sulfate-Lead Monoxide Filtrates

Plasma		Whole blood	
Urease	Hypobromite	Urease	Hypobromite
<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
19.5	19.9	16.0	16.3
9.2	8.9	18.5	18.3
12.4	12.4	22.1	21.4
15.9	15.5	17.8	17.5
15.5	15.6	36.1	34.6
13.3	13.0		
18.3	18.6		
13.5	14.0		
12.4	11.9		
19.9	20.1		
18.8	19.5		
17.3	16.9		

a need was felt for filtrates which might obviate the empirical correction for non-urea nitrogen. After trial of a considerable number of protein precipitants, it was found that a combination of ferric sulfate and lead monoxide yielded filtrates which met this need. Such filtrates are easily prepared and their urea nitrogen content estimated by the hypobromite method agrees well with urea nitrogen in tungstic acid filtrates determined by the manometric urease method of Van Slyke (2), as shown in Table I.

The reagents required are an approximately 12 per cent ferric sulfate solution and lead monoxide (litharge).

For the preparation of 1:10 filtrates, 1 volume of whole blood is laked in 8 volumes of water in a flask of at least double the final volume of the

contents. 1 volume of ferric sulfate solution is added with continuous agitation of the laked blood, followed by approximately 1.5 gm. ($\frac{1}{2}$ teaspoonful) of lead monoxide for each cc. of ferric sulfate solution. The flask is closed and shaken vigorously until a distinct metallic "clicking" sound is heard; then the contents are filtered.

For plasma, the amounts of reagents are halved, so that for 1:10 filtrates 1 volume of plasma requires 8.5 volumes of water and 0.5 volume of ferric sulfate solution.

Filtrates prepared by this method contain somewhat less nitrogen than tungstic acid filtrates and only a fraction of the original glucose. Recovery of urea added to whole blood or to plasma is shown in Table II.

TABLE II
Recovery of Added Urea

Whole blood		Plasma	
Sample	Urea nitrogen	Sample	Urea nitrogen
	<i>mg. per cent</i>		<i>mg. per cent</i>
Urea solution	9.8	Urea solution	9.5
Whole blood filtrate	20.3	Plasma filtrate	16.1
Filtrate of whole blood + urea solution	30.3	Filtrate of plasma + urea so- lution	25.8

The suggestion of Kolthoff and Sandell (3) for the extemporaneous preparation of hypobromite solutions by the addition of hypochlorite to a bromide solution has been applied to the manometric hypobromite method. It was found that a commercial bleaching solution¹ was suitable for this purpose. A mixture of equal parts of 40 per cent NaOH, 60 per cent KBr, and bleaching solution gave results equivalent to those obtained by the hypobromite reagent of Van Slyke and Kugel (1), and appeared to offer some advantage in convenience and economy.

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¹ Clorox, containing 5.25 per cent NaClO.

A PARALLELISM BETWEEN THE QUANTITATIVE INCIDENCE OF CARBONIC ANHYDRASE AND FUNCTIONAL LEVELS OF THE CENTRAL NERVOUS SYSTEM

BY WINIFRED ASHBY

WITH THE TECHNICAL ASSISTANCE OF DOROTHEA V. CHAN

(From the Blackburn Laboratory, Saint Elizabeths Hospital, Washington)

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In a previous paper (1) data were presented showing carbonic anhydrase in significant amounts in the tissue of the brain. A further study has been made of the content of carbonic anhydrase in two different regions of the central nervous system by the technique already described (2). The data here presented compare the carbonic anhydrase content of the cord with that of the cerebrum, and evaluate the findings reported with reference to the effect of differences in cellular content of the areas compared. The cord and cerebrum are chosen as widely separated "levels of function" (3) in which the cortical level is regarded as dominant over the functional level of the cord.

EXPERIMENTAL

Technique—In man the cord material was obtained upon removal of the brain and consisted of the upper cervical segments of the cord and often a fraction of the lower part of the medulla oblongata. The cord of the dogs, cats, guinea pigs, chickens, and hogs was removed from the spinal canal. The proximal halves or thirds, depending upon the size of the animal, were tested after complete removal of the meninges. The human cerebral samples were obtained by cutting off convolutions of the frontal pole. These sections contained both white and gray matter, with the gray matter predominating. In the dog, hog, cat, guinea pig, and chicken the material consisted of cerebrum, free from underlying basal ganglia. In preparation of the material for the separate determination of carbonic anhydrase of the white matter of the human frontal convolutions, the convolutions were sliced across and the gray matter was either sliced or scraped from the white fibers forming the center of the convolution. The separation of gray and white matter was not entirely complete, but approximately so.

Comparison of Carbonic Anhydrase in Cord and Cerebrum—In Table I are given comparisons of carbonic anhydrase of the spinal cord and the cerebrum in eighteen instances. In nine comparisons human autopsy material was used, eight from mental hospital patients, and one from

a normal adult male. In addition, data were obtained from two dogs, two cats, two hogs, three guinea pigs, pooled, five chickens, pooled, and a steer. In each case the carbonic anhydrase of the cerebrum was markedly greater than that of the cord. The carbonic anhydrase of the cord averaged 14.9 units per gm., that of the cerebrum 30.0 units per gm. The enzyme content of the cerebrum was approximately twice as great as that of the cord.

TABLE I

Comparison of Carbonic Anhydrase Content of Upper Cord and Cerebrum before and after Subtraction of Enzyme Attributable to Contained Blood

The human cerebral samples came from the frontal pole.

Species	Autopsy No.	Sex	Age	Carbonic anhydrase, units per gm.			
				Cord		Cerebrum	
				Total	Tissue	Total	Tissue
			yrs.				
Man.....	Normal A	M.	25	26.9	22.9	39.0	35.0
".....	7959	"	73	22.0	18.2	39.0	35.2
".....	7991	F.	82	22.0	17.7	38.5	30.9
".....	7994	M.	38	13.0	11.1	31.0	27.1
".....	7995	F.	45	15.2	7.6	25.3	17.7
".....	8026	M.	82	17.0	11.0	26.5	21.5
".....	8064	"	53	24.7	19.4	42.6	32.3
".....	8121	"	71	21.5	15.9	42.6	34.8
".....	8150	"	82	29.8	24.1	38.6	33.6
Steer.....	1	"	1½	12.1	10.1	17.7	13.7
Dog.....	1	"		17.2	14.4	44.5	39.5
".....	2	F.		13.2	8.4	29.1	25.5
Cat.....	1	"		21.9	19.5	58.8	51.7
".....	2			32.8	16.4	47.4	43.3
Hog.....	1			17.3*	13.4	37.1	33.3
".....	5	F.		18.5*	14.8	28.8	24.9
Guinea pigs...	1-3			34.8	18.1	30.8	24.7
Chickens.....	1-5			5.6	5.4	17.8	17.5

* Medulla oblongata and pons instead of cord.

Comparison of Carbonic Anhydrase of Gray and White Matter—The amount of white matter was greater than the gray matter in the cord used; on the other hand, in the human brain material the white matter was less than the gray. In the preceding comparisons of carbonic anhydrase of cord and brain it is necessary to establish whether or not the differences found may be due solely to proportionate differences in gray and white matter. In Table II is given a comparison of the content of carbonic anhydrase of the white matter, which forms the center of the cerebral

convolution, with that of the whole convolution in six instances. These were used in Table I in the comparisons of human cord and cerebrum. In each of these samples the white matter contained a greater amount of carbonic anhydrase than the mixture of gray and white forming the whole convolution. A similar separation of white matter was not attempted in the cord, but as the white matter in the cord is more abundant than the gray matter, the smaller carbonic anhydrase finding in the cord as a whole would indicate that the white matter must also have a lower carbonic anhydrase content. The inverse relationship between the ratio of carbonic anhydrase and the white matter-gray matter in the cord compared to that of human cerebral convolutions, combined with the greater content of carbonic anhydrase occurring in the white matter of the human cerebral convolutions, would rule out the possible explanation that

TABLE II

Comparison of Carbonic Anhydrase Content of White Matter within Convolutions of Human Frontal Pole with That of Combined White and Gray Matter

Autopsy No.	Carbonic anhydrase, units per gm.	
	Whole convolution	White matter within convolution
7991	30.9	36.2
7994	27.1	29.9
7995	17.7	22.8
8026	21.5	30.9
8064	32.3	37.0
Normal A	35.0	42.0

the observed differences in enzyme content of cord and cerebrum are due to differences in proportion of gray and white matter *per se*.

Furthermore in the cord of two hogs no carbonic anhydrase was found that was not attributable to the blood contained in the cord tissue. These findings are given in Table III. Since the cord of the hog may be assumed to contain approximately the same ratio between white and gray matter as that of other vertebrates, the cords of which contain an appreciable amount of carbonic anhydrase, the discrepancy cannot be accounted for on the basis of differences in number of cell bodies *versus* quantity of conducting fiber.

Correlation of Carbonic Anhydrase with O₂ Uptake Reported in Literature—Although recent work has been done by Nachmansohn *et al.* (4) on the distribution of enzymes within the neuron, work on the regional distributions of enzymes or ratios of utilization of metabolites, etc., within the central nervous system has been neglected. A small amount of earlier work is collected by Page and Quastel (5). That on oxygen utilization

bears a parallelism to carbonic anhydrase distribution found here in the instances in which the material studied is similar. In Table IV are compared the carbonic anhydrase values with the findings of Dixon and Meyer

TABLE III

Carbonic Anhydrase in Cord of Hogs

The results are given before and after subtraction of the enzyme fraction due to the blood contained in the tissue; average net content, 0.3 unit.

Hog	Section of cord	Carbonic anhydrase, units per gm.	
		Total	Tissue
A	Rostral fourth	2.3	1.0
	Second "	7.2	0.4
	Third "	2.9	0.2
	Caudal "	8.0	1.8
B	Rostral "	3.2	0.1
	Second "	2.7	-0.7
	Third "	3.0	-0.5

TABLE IV

Comparison of Carbonic Anhydrase Distribution Found in Central Nervous System of Steer and Guinea Pig with Oxygen Uptake Reported by Dixon and Meyer (Steer) and Bass (Guinea Pig) for Same Areas

Area	Carbonic anhydrase		O ₂ uptake	
Steer (37°)				
	<i>units per gm.</i>	<i>ratio</i>	<i>c.mm. per gm. per hr.</i>	<i>ratio</i>
Cerebellum.....	35.3	1.00	2550	1.00
Corpus striatum.....	24.0	0.68	1980	0.78
Cerebrum.....	13.5	0.38	1700	0.68
Thalamus.....	11.3	0.32	1170	0.46
Guinea pig (20°)				
Cerebrum.....	24.7	1.00	815.7	1.00
Cord.....	16.6	0.67	682.7	0.84

for O₂ uptake in four areas of the central nervous system of the steer (6) and of Bass for the cord and cerebrum of the guinea pig (7).

Holmes finds the relationship between rate of utilization of oxygen of the white and gray matter to be 1:3 to 1:4 (8). I have found the relationship between the carbonic anhydrase of the white and gray matter to vary greatly, depending upon the level chosen and upon the animal. In the hog

ratios of from 0:40 to 32:40 of white to gray matter may be found, while in the human, as indicated in Table I, I have found a greater content of carbonic anhydrase in the white matter than in the gray.

DISCUSSION

Since it has been shown that the greater amount of carbonic anhydrase per gm. in the functionally dominant cerebral area as compared with the cord is not dependent upon any anatomical distribution of gray and white matter, the possibility is suggested that the relationship found may be due to differences in the amount of enzyme contained in the neurons entering into the composition of the respective areas. This might be true regardless of whether the cell processes or the cell body of the neuron establishes the preponderance of the enzyme. On this basis it would seem probable that the carbonic anhydrase content might bear a relationship to the factors which cause the functional dominance of the cerebrum over the cord. As a second possibility the carbonic anhydrase may not be distributed in the neurons, but may exist in some accessory cell which may be more numerous in the cerebrum than in the cord. Dr. Stanley Cobb¹ suggested the oligodendroglia (8) as a possibility, but even so a functional significance would be conceivable.

On the other hand the greater amount of carbonic anhydrase present might not be a component of a dominant metabolism in the cerebral area but merely a result of the metabolism. We have only to assume that the carbonic anhydrase which is present in large amount in the red blood cells is readily absorbed by some constituent of the cells of the central nervous system. Then that area which has the higher metabolic rate and consequently demands the greater blood flow would in the course of time acquire the greater supply of carbonic anhydrase. One would expect that such accrued enzyme in turn might affect the metabolic level of the area in question. The parallelism between the O_2 uptake found by Dixon and Meyer and by Bass and the carbonic anhydrase content found by me is interesting but based on few data.

SUMMARY

1. The content of carbonic anhydrase in the cerebral tissue and the cord tissue has been compared. The content of the cerebral tissue was approximately twice that of the cord. Seven species of animals were studied.

2. By separate determination of the enzyme content of the white matter of human cerebral specimens it was shown that the greater amount of enzyme in the cerebrum is not due to the larger proportion of gray matter present.

¹ Personal communication.

3. The possibility of a functional relationship between the content of carbonic anhydrase and "functional levels" of the central nervous system is discussed.

I am indebted to Dr. H. Karl Langenstrass and Lieutenant-Colonel Carl Lind for brain specimens.

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THE EFFECT OF THIOUREA UPON THE METABOLISM OF IODINE BY RAT THYROID*

By ALBERT S. KESTON, E. D. GOLDSMITH, ALBERT S. GORDON,
AND HARRY A. CHARIPPER

(From the Departments of Biochemistry, College of Physicians and Surgeons, Columbia University, the College of the City of New York, and the Department of Biology, Washington Square College, New York University, New York)

(Received for publication, October 23, 1943)

Recent reports have indicated that thiourea and related compounds act as goitrogenic agents (1-4) and may be employed in the treatment of hyperthyroidism (5, 6). It has been proposed that these drugs as well as sulfonamides act by interfering with the manufacture of normal thyroid hormone (4). Franklin and Chaikoff (7) in a recent study *in vitro* have found that sulfanilamide inhibits the conversion of inorganic iodine to organically bound iodine in thyroid slices.

The present experiments were performed in an attempt to elucidate further the action of thiourea on thyroid metabolism.

EXPERIMENTAL

Adult male rats (220 to 270 gm.) were fed a ration containing 1 per cent thiourea for 2 months. At the end of this period, three animals were injected subcutaneously with 1.5 cc. of a solution of radioactive iodide ion (mainly I^{131}) containing less than 1 γ of iodine. Three normal control rats were treated similarly. 48 hours later the six animals were sacrificed, and the thyroid glands dissected and dissolved in 3 cc. of 2 N sodium hydroxide. An aliquot was removed from each for determination of radioactivity. The hydrolysate was acidified, KI and excess KIO_3 added, and the iodine extracted with CCl_4 . The radioactivity remaining in the aqueous layer is tabulated as organic I^{131} in Table I.

In a second series, immature rats (60 to 80 gm.) were employed. These were fed the thiourea ration for 17 days. Three of them and three of the normal controls were injected with 1.0 γ of radioactive iodide ion. The thyroids of these animals were removed 40 hours later, weighed, and hydrolyzed in 3 cc. of 2 N NaOH.

The iodine of the hydrolysates was then fractionated into iodide ion, thyroxine-like, and diiodotyrosine-like fractions as follows (8-10):

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The hydrolysate was made up to 20 per cent with respect to NaOH and the "thyroxine" iodine was extracted twice with an equal volume of butyl alcohol. The butyl alcohol layer was washed twice with 0.5 volume of 20 per cent NaOH and the washings combined with the aqueous layer. The

TABLE I

Distribution of Radioactive Iodine in Thyroid Glands of Normal and Thiourea-Treated Rats

The TI series represents glands taken from animals on the thiourea ration and the I series indicates glands from untreated controls. The figures given represent the values of the different fractions found in thyroid gland, expressed as a percentage of the original injected dose of radioactive iodine.

Animal No.	Weight of thyroid	Total I^{131}	Organic I^{131}
	mg.	per cent	per cent
TI-1	57	0.4	TI series pooled, < 0.03
TI-2	58	0.1	
TI-3	56	0.1	
I-4	17	8.2	I series pooled, 8
I-5	20	10.9	
I-6	15	13.5	

TABLE II

Distribution of Radioactive Iodine in Thyroid Glands of Normal and Thiourea-Treated Rats

The TI series represents glands taken from animals on the thiourea ration and the I series indicates glands from untreated controls. The figures given represent the values of the different fractions found in thyroid gland, expressed as a percentage of the original injected dose of radioactive iodine.

Animal No.	Weight of thyroid	Total I^{131}	I^{131} in iodide ion fraction	I^{131} in diiodotyrosine fraction	I^{131} in thyroxine fraction
	mg.	per cent	per cent	per cent	per cent
TI-7	18	0.1	0.06	0.016	0.003
TI-8	39	0.1	0.10	0.008	0.001
TI-9	24	0.1	0.10	0.007	0.005
I-10	7	6.3	1.0	2.7	2.5
I-11	12	6.4	1.1	2.6	2.7
I-12	7	10.5	2.5	5.4	2.5

aqueous layer was then acidified, KI and excess KIO_3 added, and the "iodide ion" iodine extracted with CCl_4 . The aqueous remainder was taken as the "diiodotyrosine" fraction. The results of these experiments are listed in Table II.

In both series the thyroids from the animals which were fed thiourea were much heavier than the thyroids from the normal animals (1-4). These

experiments reveal that 2 days after injection of radioactive iodine the thyroid glands of the thiourea-treated rats do not contain significant amounts of radioactive iodine in any form including newly formed diiodotyrosine or thyroxine. This is in contrast to the normal glands which, after similar treatment with radioactive iodine, possess considerable quantities of radioactive iodine, chiefly in organic combination.

Three experiments were conducted to ascertain the effect of thiourea on the iodine metabolism of isolated rat thyroids. In each experiment the pooled thyroid glands of five adult, normal, male rats were incubated at 38° in 5×10^{-3} M thiourea in a bicarbonate-Ringer's solution containing I^{131} with about 0.05 γ of iodide ion. Controls without thiourea were carried out at the same time. After 2 hours incubation, the glands were removed, washed twice with 2 cc. of Ringer's solution, hydrolyzed, and the radioactive iodine of the hydrolysate was separated as above into thyroxine and diiodotyrosine fractions. The results obtained in these experiments revealed that the normal controls contained organically bound radioactive iodine. The average values obtained in the control series for the diiodotyrosine and thyroxine fractions were 2.7 and 0.9 per cent, respectively, of the total I^{131} . The glands incubated with thiourea contained relatively little organically bound radioactive iodine. The average values obtained in the thiourea series for the diiodotyrosine and thyroxine fractions were 0.16 and 0.015 per cent, respectively, of the total I^{131} .

These studies thus constitute experimental evidence for the statement that thiourea interferes with the normal production of thyroid hormone by the thyroid gland.

SUMMARY

Radioactive iodine was administered to young and adult normal and thiourea-fed rats. Analysis of the thyroid glands of the thiourea-treated rats, 48 hours after the injection of the iodine, revealed no appreciable amounts of radioactive iodine in any form, whereas thyroid glands from normal rats contained considerable quantities of radioactive iodine, chiefly in organic combination. Experiments *in vitro* revealed no substantial formation of organically bound radioactive iodine in thyroid glands incubated with thiourea.

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THE ESTIMATION OF ALLANTOIN IN BLOOD

By E. GORDON YOUNG, CATHERINE CONWAY MacPHERSON, HELEN P. WENTWORTH, AND WINTHROP W. HAWKINS

(From the Department of Biochemistry, Dalhousie University, Halifax, Canada)

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The determination of allantoin in blood has not been practicable as a routine procedure in the past because of the relatively large amounts of allantoin required for an estimation. Hunter (8) was the first to prove the presence of allantoin in mammalian blood when he obtained amounts of 0.78 mg. per 100 ml. for the ox and 0.57 mg. for the pig. These values were established by isolation of allantoin from 800 ml. of blood and are only semiquantitative in character. Fosse *et al.* (6) have applied their spectrophotometric technique to the estimation of allantoin by the Rimini-Schryver reaction, using 5 ml. of serum deproteinized by trichloroacetic acid. They have recorded values of 1 to 2.7 mg. for the ox, calf, horse, sheep, and pig.

In our studies of purine metabolism the need for a simple colorimetric procedure for allantoin in blood was soon appreciated. Consequently we have attempted over several years to apply the technique of the Rimini-Schryver reaction as developed in this laboratory (10) to the estimation of allantoin in blood with a Klett colorimeter. The method depends upon the hydrolysis of allantoin to allantoic acid and this to glyoxylic acid. The latter is estimated on the basis of a sensitive color reaction with phenylhydrazine and potassium ferricyanide.

EXPERIMENTAL

The direct application of the Rimini-Schryver reaction to blood filtrates deproteinized by tungstic acid was not possible because the color developed was too weak for comparison with a standard solution of potassium allantoate. The presence of glucose in relatively large quantity introduced a further difficulty owing to osazone formation with phenylhydrazine. The problems presented in the application of the Rimini-Schryver reaction to blood were (1) the induction of the phenylhydrazine reaction with the glyoxylic acid produced by the acid hydrolysis of allantoic acid in preference to that with glucose, and (2) the removal of protein without undue dilution.

As recorded in a previous paper (10), the former difficulty can be overcome by separating the hydrolysis of allantoic acid carried out at 100° in 0.02 N hydrochloric acid from the reaction of the glyoxylic acid so formed with phenylhydrazine. The latter reaction can then be carried on at a temperature 25–30°.

To overcome the second difficulty two deproteinizing agents have been suggested, trichloroacetic acid by Fosse *et al.* (5) and uranium acetate by Florkin and Bosson (3). With solid trichloroacetic acid to a concentration of 20 per cent in the blood sample, it was possible to develop a color in the filtrate comparable with the standard. The color, however, was frequently off shade and faded much more rapidly than the standard, passing through the maximum in less than 5 minutes. Trials with trichloroacetic acid added to the standard showed that its presence decreased the intensity of the maximum color by over 60 per cent. It was therefore rejected as a deproteinizing agent.

Uranium acetate was not found to be suitable, as dilution of the blood sample was necessary and the yellow color of excess precipitant interfered in the colorimetry.

Tungstomolybdic, phosphotungstic, and sulfosalicylic acids were tried. Sulfosalicylic acid gave the best results and interfered with the standard reaction only to the extent of making the red shade pinker. The necessity for careful neutralization of the protein-free filtrate is an undesirable feature of this reagent.

Following the procedure of Deutsch, Eggleton, and Eggleton (2) who used anhydrous sodium sulfate in the preparation of protein-free tissue extracts, we have applied this reagent with satisfactory results to diluted blood. The technique adopted is as follows:

Visual Colorimetric Method

Reagents—

1. Sodium sulfate, anhydrous solid.
2. Sodium hydroxide, 1 N.
3. Hydrochloric acid, 1 N and concentrated.
4. Phenylhydrazine hydrochloride. Recrystallize from 98 per cent ethyl alcohol, wash with absolute alcohol, and dry over potassium hydroxide in a desiccator in the dark. 0.05 gm. in 15 ml. of water; prepare daily prior to use.
5. Potassium ferricyanide. Recrystallize twice from water and dry at 50° (7). 0.25 gm. in 15 ml. of water; prepare daily.
6. Potassium allantoate standard, 20.0 mg. in 1000 ml. of 0.01 N sodium hydroxide; prepare every 3 months. For preparation of potassium allantoate see Young and Conway (10).

*Procedure—*Pipette 5 ml. of oxalated whole blood into a 50 ml. beaker, add 10 ml. of distilled water, and warm to 40° on a water bath. Then add 8 gm. of anhydrous Na_2SO_4 , a small quantity at a time while stirring with a mechanical stirrer. Stir continuously at the same temperature for about

30 minutes. Filter the mass with suction as rapidly as possible through a 2.5 inch Buchner funnel, shown as *C* in Fig. 1, fitted with a Whatman No. 5 circle, first moistening the edge of the paper with a few drops of the mixture. The apparatus must be kept warm by immersion in a water bath at 40°.

Pour the filtrate into a glass tube, 1 × 3 inches, with a small hole in the bottom plugged with a fragment of rubber, *D* (Fig. 1). Cool the filtrate in an ice-water bath at 0°. Stir the mixture occasionally as the $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ crystallizes. The whole tube should be filled with a crystalline paste if the yield of filtrate is to be adequate. Keep the tube at 0° until ready to centrifuge.

The plug is now removed and the tube is wiped dry. It is encased with Gooch rubber tubing and placed in a 50 ml. centrifuge tube with indented sides, or a tapered one, which will allow space for about 5 ml. of centrifugate, shown as *B* in Fig. 1. The tube should be well cushioned in the trunnion cup. It is spun at about 1000 R.P.M. for 3 to 5 minutes. This should pro-

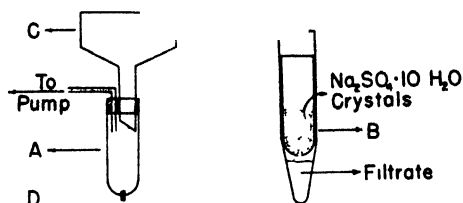


FIG. 1. Filtration apparatus. *C*, Buchner funnel, *A*, test-tube; *D*, rubber plug; *B*, centrifuge tube.

duce about 3.5 ml. of clear fluid. The centrifuge tube should be immersed in the ice bath again for about 5 minutes to be sure that no further $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ will crystallize at 0°. If it does, it may be centrifuged down and the fluid decanted.

Pipette an aliquot of 1 ml. into small test-tubes, 0.5 × 3 inches, graduated at 3 ml. Add 0.10 ml. of 1 *N* NaOH and incubate at 37° for 12 hours. Then add 0.15 ml. of 1 *N* HCl. Pipette 1 ml. of standard potassium alantoate into a similar tube and add 0.05 ml. of 1 *N* HCl. All tubes are now placed in a boiling water bath for *exactly* 2 minutes and then transferred to an ice-water bath. To each tube add 0.2 ml. of phenylhydrazine hydrochloride solution, mix well, and place in bath at 30° for 15 minutes. Transfer to an ice-salt bath at -10° till incipient freezing. Add to each tube 0.6 ml. of concentrated HCl, previously cooled to -10°, and 0.2 ml. of $\text{K}_4\text{Fe}(\text{CN})_6$ solution. Shake the tubes and allow 30 minutes for full development of color. Fill to the mark with water and compare in a colorimeter with micro cups and plungers.

Calculation—

$$\frac{R_s}{R_u} \times C_s \times 0.738 \times \frac{100}{v} \times \frac{d}{c} = \text{mg. allantoin in 100 ml. blood}$$

- where R_s = reading of standard
 R_u = " " unknown
 C_s = mg. potassium allantoate in 1 ml. standard
 v = volume of filtrate used
0.738 = factor for converting allantoate to allantoin
 d = degree of dilution
 c = factor " concentration

As set down above this usually becomes

$$\frac{R_s}{R_u} \times 0.02 \times 0.738 \times \frac{100}{1} \times \frac{3}{2.5}$$

Concentration Ratio—The degree of concentration brought about by the sodium sulfate is an important factor in the accuracy of the method. The

TABLE I
Concentration Ratio for Allantoin in Pure Solution

Allantoin	C_s	C_u	Concentration ratio
<i>mg. per ml.</i>	<i>mg. per ml.</i>	<i>mg. per ml.</i>	
0.015	0.01	0.0143	2.1
0.020	0.02	0.0191	2.1
0.020	0.02	0.0200	2.2
0.030	0.02	0.0400	1.9
0.030	0.02	0.0428	2.1
Average.....			2.1

anhydrous salt passes slowly to the hydrated form at or below 33° (Berkeley (1)). 9 parts of anhydrous salt absorb 11 parts of water, leaving 4.7 per cent solution at 0°. It was first emphasized as a protein precipitant under these conditions by Pinkus (9) and as a means of concentration by Deutsch, Eggleton, and Eggleton (2). The degree of concentration for dilute aqueous solution is given by them as 2.5 and for tissue extracts with appreciable total solids present in solution as 2.6 to 3.2, depending on the constituent measured.

To determine the concentration factor for allantoin under our conditions pure aqueous solutions were first tested by the above technique with 5 ml. of solution of known concentration. The results are recorded in Table I, showing good agreement with an average of 2.1 instead of 2.5 as calculated on theoretical assumption. The factor 2.5 was obtained, however, in a series of determinations with potassium allantoate in a range of concentrations from 0.5 to 10 mg. per 100 ml.

We next attempted to determine the concentration factor for other constituents of blood under our experimental conditions. These are given in Table II and they show considerable differences.

Creatinine, glucose, and allantoin were combined in one solution and the concentration ratios determined. They still showed a difference between different constituents of the original solution, with allantoin at 2.3, creatinine at 2.6, and glucose at 2.8. This suggests the importance of adsorption and the physical state of the crystalline mass.

Comparisons of the non-protein nitrogen were next made on three protein-free blood filtrates obtained by (1) tungstic acid and (2) Na_2SO_4 precipitation calculated on a concentration ratio of 2.5. 39.9, 39.9, and 44.4 mg. per 100 ml. of blood were obtained with the tungstic acid filtrate, and 41.8, 40.0, 47.2 mg. per 100 ml. of blood with Na_2SO_4 . These figures would suggest that the factor used was approximately correct.

TABLE II
Concentration Ratios for Other Blood Constituents in Pure Solution

Substance	Method	C_s	C_u	Concentration ratio
		mg. per 100 ml.	mg. per 100 ml.	
Urea.....	Folin-Svedberg	49.3	24.3	1.5
Glucose.....	Folin-Wu	10.0	9.7	2.9
".....	"	12.3	11.1	2.7
Creatinine.....	Folin	100	77.8	2.3

Since the presence of protein might exert an effect on the concentration ratio, it was desirable to test the behavior of allantoin alone added to such a solution. A 20 per cent ovalbumin solution was tested with 5 ml. and an allantoin solution containing 0.125 mg. added in place of the water of dilution. Although there was a slight amount of allantoin found in a blank determination, this was not large enough to disturb the final values which averaged 2.4 for the concentration ratio. The method was next applied to a "synthetic" blood made up as follows (per cent composition): hemoglobin 13.00, serum albumin 6.00, glucose 0.100, urea 0.060, creatinine 0.001, NaCl 0.900, KCl 0.030, CaCl_2 0.025, NaHCO_3 0.020, $\text{MgHPO}_4 \cdot 3\text{H}_2\text{O}$ 0.017, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.004. The results are recorded in Table III, showing the recovery of added allantoin as above with a concentration factor of 2.1 and 2.5.

The method was next applied to fresh defibrinated dog and cat blood, as shown in Table IV, with a concentration ratio of 2.5 in calculation. In other single determinations on various specimens of dog blood we have obtained values of 1.2, 1.3, 1.6, 1.6, 1.7.

The figures in Table IV and as given above show that the normal fluctuations of allantoin in dog blood are between 0.8 and 1.7 mg. per 100 ml. The one figure of 7.8 suggests some abnormality. The figures for cat blood seem to be somewhat higher. We have also followed for 1 week the daily fluctuation in the blood allantoin of two ordinary dogs fed Purina dog chow. The range was between 1.2 and 2.3 mg. for both animals. Our values therefore agree in order of magnitude with those given by Fosse, Brunel, and Thomas (6) at 1 to 2.7 mg. for serum in the ox, calf, horse, sheep, and pig as determined photocolrimetrically.

TABLE III
Recovery of Allantoin Added to Synthetic Blood

Allantoin added mg. per 100 ml.	Allantoin concentration		Recovery	
	2.5 mg. per 100 ml.	2.1 mg. per 100 ml.	2.5 per cent	2.1 per cent
0	0.92	1.10		
2.0	2.68	3.19	88	104
4.0	4.20	5.00	82	97

TABLE IV
Recovery of Allantoin Added to Defibrinated Blood

Animal	Original	Added	Total	Recovery	
	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg.	per cent
Dog 1.....	1.11	2.0	3.10	1.99	100
" 2.....	0.82	2.0	2.88	2.06	103
" 3.....	0.92	3.0	4.56	3.64	121
" 4.....	7.80	2.56	10.18	2.38	93
Cat 1.....	2.40	2.56	4.89	2.49	97
" 2.....	1.09	4.56	4.58	3.49	77
" 3.....	3.17	2.56	5.60	2.43	95

As a pure bred Dalmatian coach hound was available, our interests led us to determine the blood allantoin. If present in quantity, uric acid would interfere with the allantoin estimation; consequently the latter was determined by Folin's direct procedure (4) after deproteinization with sodium sulfate. The values are recorded in Table V, and it is interesting to note that the level of allantoin in Dalmatian blood is lower than in ordinary dog blood. The level of uric acid would appear to be about the same as in the ordinary dog.

Although there is still some uncertainty as to the correct concentration factor to be used in calculation, the method as outlined provides a simple

technique for the estimation of allantoin in blood without the use of a spectrophotometer. It is applicable to metabolic studies and has thus been used for several years in this laboratory. The accuracy of the method is probably about ± 10 per cent but the error at times has appeared to be greater. Duplicate determinations agree well within themselves.

TABLE V
Purines in Blood of Dalmatian Coach Hound

Date	Allantoin	Uric acid
<i>Mar.</i>	<i>mg. per 100 ml.</i>	<i>mg. per 100 ml.</i>
18	0.42	0.24
19	0.23	0.16
20	0.45	0.12
21	0.50	0.17
Average.....	0.40	0.17

TABLE VI
Effect of Tungstate on Optical Density

Allantoin <i>mg.</i>	Aqueous preparation		Tungstate preparation	
	Optical density $\times 10^3$	Optical density — blank $\times 10^3$	Optical density $\times 10^3$	Optical density — blank $\times 10^3$
0	4	0	7	0
0.00025	17	13	18	11
0.0005	24	20	28	21
0.00075	37	33	40	33
0.001	42	38	45	38

Photocolorimetric Method

Since the main difficulty in the visual colorimetric method was one of excess dilution, the application to a photocolorimetric method was obviously a solution. We have used the Lumetron photoelectric colorimeter, model 401, manufactured by the Photovolt Corporation of New York. Blood proteins were precipitated by the Folin-Wu method with sodium tungstate and sulfuric acid with 1 ml. of blood. The mixture was well shaken and allowed to stand for 15 minutes before filtration. Aliquots of 1 ml. were treated exactly as described for the centrifugate in the method given above, except that the final volume of dilution was 5 ml. instead of 3 ml.

The greatest optical density was obtained at 520 $m\mu$ wave-length with a potassium allantoate standard and a series of filters. The region of greatest absorption, however, extended between 450 and 550 $m\mu$. The standard

B-530 $m\mu$ Corning filter supplied with the instrument was found suitable for routine estimations. The Rimini-Schryver reaction has previously been shown to obey Beer's law and this was again confirmed for the lower limits of color with about 0.5 γ of allantoate.

The effect of the presence of tungstic acid on the optical density was next investigated. Table VI shows that in the form of its sodium salt it does not affect the results. 5 ml. portions of the allantoin solution were diluted in two series to 50 ml. with water and with tungstic acid as in the regular procedure. 25 ml. of this solution were then neutralized with NaOH and diluted to 50 ml. with water. 1 ml. was used for a determination. Most of the tungstic acid, however, is removed by the blood proteins, so that neutralization is not necessary when the method is applied to blood.

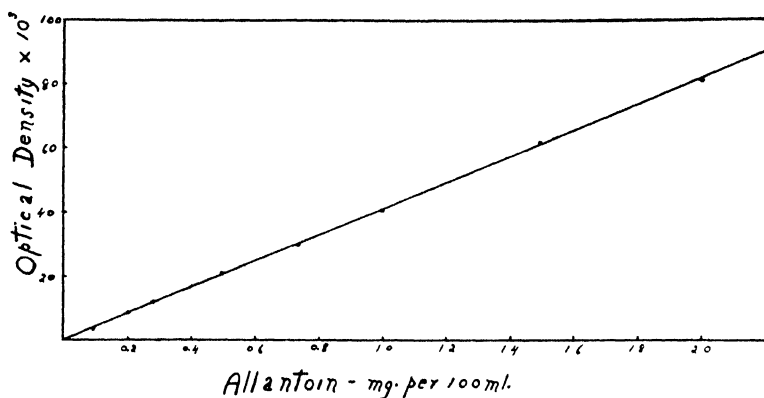


FIG. 2. Photocolorimetric reference curve of the reaction in pure solution

Standard reference curves on solutions of pure allantoin were next prepared. Fig. 2 is a typical result of plotting optical density minus the blank against concentration in mg. per 100 ml. The result was always a straight line but the optical density of the blank varied with each experiment and the corresponding values for the allantoin varied accordingly. The greatest variation was apparent at a concentration of 2 mg. per cent. It is therefore recommended that standards be run along with the blood filtrates.

Several determinations were carried out on blood filtrates to which allantoin had been added. Thus one specimen of dog blood had initially 1.28 mg. per 100 ml. After 1 mg. of allantoin was added, 2.33 mg. were obtained, a recovery of 105.6 per cent. Another specimen had initially 2.26 mg. After addition of 2 mg. of allantoin it showed 4.26 mg. or a recovery of 100 per cent. It is thus possible to apply the method to the photoelectric colorimeter with satisfactory results.

SUMMARY

A visual colorimetric method for the estimation of allantoin in blood based on the Rimini-Schryver reaction with an approximate accuracy of ± 10 per cent has been developed, requiring 5 ml. of blood. Sodium sulfate is used as a protein precipitant and concentrating agent. The concentration ratio for allantoin has been investigated.

A photoelectric colorimetric method has also been developed with 1 ml. of blood with tungstic acid as protein precipitant.

Allantoin has been found to vary between 0.8 and 2.3 mg. per 100 ml. of dog blood. In the Dalmatian the average concentration is 0.4 mg.

We wish to express our thanks and indebtedness to the Banting Research Foundation for financial assistance during the course of this investigation. Grateful acknowledgment is also made to Dr. H. B. Collier for advice in the development of the photocolorimetric method.

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MICROESTIMATION OF THE INORGANIC CONSTITUENTS OF BONE

By ALBERT E. SOBEL, MORRIS ROCKENMACHER, AND
BENJAMIN KRAMER

(From the *Pediatric Research Laboratory and Division of Biochemistry, The Jewish Hospital of Brooklyn, Brooklyn, New York*)

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This paper reports a scheme of bone analysis for small samples which need not be in powder form. It was devised to analyze the small bones of low ash content which were encountered in experiments on the composition¹ of bone in young rats on experimental diets which produced rickets and poor growth. Such bones, containing relatively large amounts of organic matter, are difficult to reduce to powder, which is necessary for most methods of carbonate analysis.

The design of this scheme permits the various analyses to be made easily and precisely in a serial fashion on a single specimen weighing as little as 5 mg. The decomposition of the carbonates in the specimen for the determination of carbonate results in an HCl solution of the bone sample. This solution is made to a definite volume and aliquot portions are used for the other analytical procedures. Time is saved, since only one weighing is required and the carbonate analysis and the solution of the bone are accomplished simultaneously.

Calcium may be determined in quantities as minute as 10 γ with an error of ± 0.1 γ (1 per cent), phosphorus in even smaller amounts with equal precision, 35 γ of carbonate carbon to 1 part in 1000 (as little as 3.5 γ of carbonate carbon to 1 part per 100) (1-3), and concentrations of lower than 0.02 milliequivalent of total base with less than a 2 per cent error. With larger amounts of sample, the precision of the methods increases.

This procedure may also be used in analyzing the individual teeth of young rats, urinary calculi, and other calcified structures when only small samples are available.

Preparation of Bone

The bone is removed from the animal and dissected clean of all adhering soft tissue. Slits are made longitudinally through the diaphysis and epiphyses to render extraction more effective. The bone is extracted twice

¹ A report partially presented before the Division of Biological Chemistry at the 104th meeting of the American Chemical Society at Buffalo, September 7-11, 1942 (see also *Federation Proc.*, 2, 70 (1943)).

with alcohol and once with ether for overnight periods. The bone is dried at 105° overnight in an electric oven. At this stage, either sections of the bone are removed for analysis or the entire bone is analyzed without the necessity of powdering. Previous to weighing, the samples are dried at 105° for an hour and cooled to room temperature in a desiccator.

Determination of Carbonate

Carbonate is determined by a modification of the manometric carbon method reported by Van Slyke *et al.* (1, 2). In the procedure described below, the sample is subjected to decomposition with HCl and not to combustion with an oxidizing solution.

The apparatus and the principles of the method are described by Van Slyke and Folch (2). Certain details of the procedure and apparatus are of necessity modified for our purposes. By incorporating MacFadyen's (3) modification for preparing the reagents, it is possible to measure the pressure of the evolved CO₂ at 0.5 cc. volume and to estimate even more precisely micro quantities of carbonate.

Apparatus—The apparatus is diagrammed in Figs. 1 to 5 of Van Slyke and Folch (2). Except for the addition of a water-cooled condenser around connecting tube *Q* (Fig. 1), the apparatus and accessory equipment are that of Van Slyke and Folch. The water jacket serves to condense vapors produced during the decomposition, preventing them from blocking connecting tube *Q* and from entering the manometric chamber *C*, where they would change the volume of the absorbing alkali solution.

The decomposition tubes are made of Pyrex with a 14/35 standard taper female joint to fit the 14/35 male joint on the water-jacketed tube.² For the sake of correct terminology, the combustion tubes of Van Slyke are referred to here as decomposition tubes.

The micro burner and the connecting tube with its fitted decomposition tube are all clamped to a single ring-stand.

For purposes of clarity and reference, the symbols and designations applied by Van Slyke and Folch (2) are retained in the discussion.

Reagents—

3 N HCl. Redistilled HCl is appropriately diluted to 3 N $\pm$ 0.2 N.

CO₂-free, approximately 0.5 N NaOH.³ A stock solution of concentrated alkali is prepared by dissolving c.p. NaOH in an equal weight of distilled

² Water-jacketed connecting tube and decomposition tubes made by Eimer and Amend, New York.

³ MacFadyen (3) has reported that by preparing the 0.5 N NaOH and the 2 N lactic acid reagents in 25 per cent NaCl solution the gas may be measured at the 0.5 cc. volume to give results precise to 1 part per thousand. This procedure is recommended for general use and particularly for micro samples.

water; it is stored in a paraffin-lined, rubber-stoppered bottle protected by soda lime. The carbonate is permitted to settle out. To prepare the 0.5 N alkali, 3 cc. of the clear, carbonate-free, concentrated NaOH solution are mixed with 100 cc. of distilled water which has just been freed of CO_2 by adding 1 drop of 0.1 N HCl and boiling. The solution is stored in the soda lime-protected cylinder ((2) Fig. 3, *D*) which is provided with a rubber tip.

5 N NaOH solution. 30 cc. of stock, saturated NaOH solution are diluted to 100 cc. with distilled water and stored in a cylinder ((2) Fig. 3, *D*) without a rubber tip.

2 N lactic acid.³

Sampling—The sample (powder, section, or whole bone) is weighed by difference on a counterbalanced scoop as described by Van Slyke and Folch ((2) Fig. 5). A number of samples for a day's analysis are weighed out and transferred to their respective decomposition tubes, which are closed with rubber stoppers and placed upright in a wire basket until analyzed.

Determination of CO_2 —Several bones can be decomposed simultaneously if enough apparatus is available. When ready, caprylic alcohol (5 drops) is added to the sample to reduce foaming, and the decomposition tube *T* is fitted into the connecting tube *Q*. The connecting tube is joined glass to glass by heavy walled rubber tubing with the side arm of the manometric chamber *C*, which is full of mercury ((2) Fig. 1). The decomposition tube and the connecting tube are clamped securely onto the ring-stand and water is circulated through the condenser on connecting tube *Q* (Fig. 1).

The technique of the analysis from here on follows exactly that of Van Slyke and Folch, up to the point where these authors add the chromic acid oxidizing mixture. Instead of this a measured volume of 3 N HCl is pipetted into cup *F* and run into *T*, and the cock here is closed. The volume of HCl used depends on the size of the sample and the volume to which the HCl solution is to be made prior to subsequent analysis. In this work, 5 cc. of the acid were found convenient. The micro burner is brought under tube *T* and the tube is heated. Bubbles of CO_2 from the decomposing bone will rise through the HCl. Heating must be gentle to avoid bumping the HCl into the connecting tube *Q* and chamber *C*. As decomposition proceeds, the mercury will fall somewhat in *C* and rise slightly in the manometer. The evolved CO_2 is absorbed by the alkali in *C*. The rise of mercury in the manometer is due to the vapor pressure of water and to the expansion of the residual unabsorbed gases in the system when the tube is heated. Should the mercury reach the top of the manometer, it indicates either insufficient preliminary elimination of atmospheric gases, leaking apparatus, or CO_2 in excess of the amount which can be absorbed by the alkali in *C*.

Heating is continued for 10 to 15 minutes, until decomposition is complete. Any particles of bone which are mechanically carried up the tube out of reach of the HCl can be washed back into the acid by shaking the tube. If whole bone is used, the time required for decomposition may be reduced by breaking the weighed bone into fragments in the decomposition tube. This operation may be accomplished without loss by means of a stiff glass rod with fire-polished ends, passed through a cork fitted into the decomposition tube.

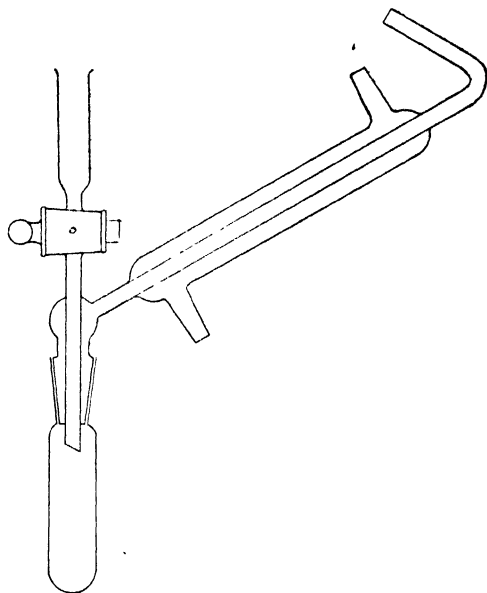


Fig. 1. Diagram showing decomposition tube and connecting tube *Q* with water-cooled condenser.

Decomposition is complete when the bone dissolves completely except for a few isolated particles of insoluble organic matter. The flame is removed and the mercury in *C* is lowered to the 50 cc. mark and raised to about the 10 cc. mark by alternately lowering and raising the leveling bulb *L* with cock *a* open. Twenty to twenty-five repetitions of this manipulation serve to transfer the evolved CO_2 quantitatively into the absorption alkali in chamber *C*. An alternate procedure for accomplishing this operation is described by Van Slyke ((2) Fig. 4, *B*). Cock *b* is then closed, the condenser water shut off, and connecting tube *Q* disconnected from the side arm of *C*.

The CO_2 absorbed by the 0.5 *N* NaOH in the chamber is then determined as described by Van Slyke and Folch.

A blank is usually run routinely once or twice a week and whenever new reagents are prepared. For the blank analysis, 5 drops of caprylic alcohol are placed in an empty decomposition tube and the procedure is carried through exactly as described above. The blank is usually set up first and the bone samples weighed out during the boiling stage.

The HCl solution of the bone, to be used for the analysis of other elements, is quantitatively transferred through Whatman No. 42 filter paper into a volumetric flask and made to a convenient volume, depending on the amount of sample taken and its composition. The connecting tube *Q* and decomposition tube *T* are rinsed with distilled water from a wash bottle and these washings added to the solution in the volumetric flask. The clear solution in the flask is well mixed and aliquot portions of this are

TABLE I
Estimation of Carbonate

Liquid standard; 1 cc. = 100 volumes per cent CO₂

Solid standard: 100 mg. = 2.00 mg. CO₂

99.8	2.00
100.0	1.98
100.1	1.98
100.0	1.99
99.7	2.00
99.8	1.97
99.9	1.99
99.7	1.99
99.9	1.99
99.9	2.00

used for the subsequent analysis of Ca, P, total base, and NH₄⁺. In these investigations the solution was usually made to 50.0 cc.

Calculations—

$$\text{Mg. CO}_2 \text{ in bone} = P_{\text{CO}_2} \times \text{factor CO}_2$$

$$P_{\text{CO}_2} = p_1 - p_2 - c$$

$$c = p_1 - p_2 \text{ for blank analysis}$$

The factors for calculating results in terms of carbon are given by Van Slyke and Folch (2). These are multiplied by 4.997 to give the mg. of CO₂ and by 3.664 to give the mg. of CO. (If the NaCl-saturated solutions of MacFadyen (3) are used, the factors should be taken from his tables.)

The procedure was first checked with both liquid and solid carbonate standards. The solid carbonate standards were made up by mixing thoroughly with a mortar and pestle a weighed portion of sodium carbonate with a greater weight of NaCl. A known weight of the mixture was taken for analysis. The results appear in Table I. The liquid standards show

the high degree of precision attainable. The results with the solid standards probably suffer from the difficulty in preparing the mixture of a small

TABLE II
Composition of Bone

In Determination I one femur from each rat was analyzed by the authors' micro-procedures for Ca and P. In Determination II one femur from each rat was analyzed by gravimetric macroprocedures (5) for Ca and P.

Litter and dietary group	Determination I					Determination II*				
	Weight of dry bone	CO ₂	Ca	P	Residual Ca:P†	Weight of dry bone	CO ₂	Ca	P	Residual Ca:P†
	mg.	per cent	per cent	per cent		mg.	per cent	per cent	per cent	
I (1st day).....	43.6	2.08	15.8	6.96	2.08	43.9	1.95	15.3	6.83	2.03
K-A.....	70.2	2.03	12.3	5.74	1.90	71.6	2.11	11.9	5.58	1.89
G-B.....	91.4	3.55	16.9	7.65	1.90	95.1	3.87	17.2	7.48	1.95
I-A + vitamin D	76.0	1.83	12.2	5.77	1.92	76.2	1.83	12.7	5.94	1.93
I-B + vitamin D	107.5	4.06	18.0	7.82	1.96	108.2	4.13	18.5	7.88	2.00
K-C + vitamin D	82.2	2.20	14.4	6.56	1.97	79.2	2.32	14.0	7.20	1.93
H-C†.....	62.1	2.24	13.8	7.40	1.68	66.7	2.38	13.5	6.88	1.73
I-C†.....	57.7	2.13	12.1	6.58	1.63	62.3	2.20	12.3	6.28	1.69
J-C†.....	58.1	2.47	12.0	6.18	1.71	60.5	2.43	12.4	6.06	1.77
K-C†.....	67.1	2.30	12.8	6.97	1.62	70.0	2.27	12.4	7.12	1.54
Mean§.....		2.489	14.03	6.763	1.837		2.549	14.02	6.725	1.846
Absolute amounts of component in sample, mg.....							0.9-4.46	0.7-20	3.0-8.5	

* The entire femur was used for carbonate estimation and the solution obtained containing the Ca and P of the entire bone was used for the subsequent gravimetric procedure.

† $\frac{\text{Total Ca} - (\text{Ca combined with CO}_2)}{\text{Total P}}$ (weight ratio) (6).

‡ The bones whose composition is shown in this table are from experiments which are prepared for publication. The low values of the residual Ca:P ratios in dietary Group C will be discussed in a subsequent paper.

§ P, the probability that the difference between two means is due to chance, is 0.32 for CO₂, 0.93 for Ca, 0.75 for P, and 0.59 for residual Ca:P calculated by the statistical method of Fisher (4) as applied to small samples.

amount of sodium carbonate with a larger amount of NaCl homogeneously and without loss.

Another type of check was performed by comparing the results obtained from the analysis of both the right and left femurs of an animal. The precision obtained is apparent from an inspection of Table II, from which

it is seen that the slight difference between the mean values is without statistical significance (4).

The question arose during the course of the experiment as to the possibility of decarboxylation of some of the organic components of the bone during boiling with HCl. Such an occurrence would result in increased CO₂ values. To investigate this possibility, samples of bone powder were analyzed by the technique described here and by the method of Shear and Kramer (6). The latter method cannot be subjected to the criticism expressed above because decomposition takes place at room temperature. In Table III, the values obtained by the two methods are compared. It is readily seen that the objection is not substantiated by the data.

TABLE III

Comparison of Results of Carbonate Analysis of Bone by Methods of Shear and Kramer (6) and Authors

All analyses were performed on the same bone.

Method	Weight of sample	CO ₂	CO ₂
	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
Shear and Kramer	11.7	0.203	1.74
	43.5	0.738	1.69
	36.2	0.619	1.71
	21.1	0.358	1.70
	27.3	0.470	1.72
	19.2	0.321	1.67
Authors	20.3	0.328	1.62
	19.7	0.311	1.58
	27.9	0.447	1.60
	38.4	0.609	1.59
	31.3	0.501	1.60
	24.7	0.396	1.60

With 0.14 mg. of carbonate carbon, a precision of 1 part per 1000 is possible when the gas is measured at the 2.0 cc. volume. This is the quantity found in 15 to 60 mg. of bone, depending on the carbonate content. Such amounts were used in our experiments in which this scheme was employed and consequently our reagents were prepared in distilled water.

One-fourth of the above amounts may be determined by using MacFadyen's (3) reagents, which enable the pressure of the gas to be measured at the 0.5 cc. volume with similar precision.

Determination of Calcium

Calcium is determined on aliquot portions of the solution by the direct acidimetric method of Sobel and Sklersky (7) with the indicator mixture

described by Ma and Zuazaga (8). For smaller samples, the modification described by Sobel and Sobel (9) is used. The apparatus and reagents are those described in the above papers except that Ma and Zuazaga's indicator replaced Patterson's indicator. This consists of 10 parts of a 0.1 per cent alcoholic solution of brom-cresol green with 2 parts of a 0.1 per cent alcoholic solution of methyl red. For the submicromethod, this is diluted 1:10 with distilled water. The color of the indicator is green in alkaline solution and goes through a colorless stage to a salmon-pink at a lower pH.

Procedure—Transfer 1 or 2 cc. of bone solution to a 15 cc. Pyrex, conical centrifuge tube and add 2 cc. of saturated ammonium oxalate solution. Adjust the solution to the yellow of thymol blue (pH 3.0 to 4.0) with dilute ammonia and proceed as described by Sobel and Sklersky (7). A known amount of calcium standard solution is analyzed with each set of determinations of bone calcium as a check on reagents and technique.

TABLE IV
Determination of Calcium in Presence of Phosphate

The values are expressed in mg.

P added	Ca found*	P added	Ca found*
None	0.200	0.400	0.202
"	0.200	0.400	0.200
"	0.202	0.400	0.200
"	0.198	0.400	0.198
0.400	0.200	0.400	0.196

* Ca present = 0.200 mg.

By the use of conical centrifuge tubes of only 4 cc. capacity, a Rehberg capillary micro burette, and by reducing the volumes to one-tenth and diluting the indicator solution 10 times, as little as 10 γ of calcium may be estimated in 0.1 cc. of solution (Sobel and Sobel (9)).

Calculation—

Mg. Ca in bone sample

$$= \frac{\text{cc. acid} \times \text{normality of acid} \times 20 \times \text{total volume of bone solution}}{\text{volume of bone solution employed in analysis}}$$

McComas and Rieman (10, 11) show that under conditions similar to those employed here only 0.0005 mm of calcium remains unprecipitated in 200 cc. of solution at pH 3.7 containing 8 mm of excess oxalate. If these data are applied to our procedure, only 0.001 mg. of calcium or 0.0001 mg. in the submicromethod remains unprecipitated from the bone solution analyzed. The work of these authors corroborates the statement of

Washburn and Shear (5) that a pH of 3 to 4 is optimal for precipitating calcium as the oxalate.

The method was checked by analyzing known calcium solutions. The interference of phosphate in our procedure appears to be without significance according to the data obtained and presented in Table IV. The average error of duplicate estimations was within 1 per cent. The right and left femurs of a number of experimental animals were analyzed by the gravimetric procedure of Washburn and Shear (5) and the values obtained compared favorably with those found by the acidimetric procedure as shown in Table II.

Determination of Phosphorus

The estimation of phosphorus is performed on aliquot portions of the bone solution by an adaptation of the Fiske-Subbarow method (12).

Reagents—These are prepared as described by Fiske and Subbarow (12).

2.5 per cent ammonium molybdate solution in approximately 0.5 N sulfuric acid (Molybdate I).

0.25 per cent 1-amino-2-naphthol-4-sulfonic acid solution. Filter if necessary and store in a dark bottle away from the light. Prepare fresh every month, or when the solution has a yellow appearance.

Stock phosphorus standard solution. 1 cc. contains 0.008 mg. of P.

Procedure—Transfer 0.1 or 0.2 cc. of bone solution to a Kramer-Gittleman tube graduated at 2 cc. Add 0.2 cc. of Molybdate I and 0.1 cc. of the aminonaphtholsulfonic acid solution. Dilute to the mark, mix, and read against a simultaneously prepared standard in a Bausch and Lomb Duboscq micro colorimeter after 15 to 20 minutes. The bone solution is diluted if the color is too intense. The standard is prepared simultaneously by transferring 5 cc. of the phosphorus working standard (5 cc. $\approx$ 0.04 mg. of P) to a 10 cc. glass-stoppered volumetric flask. Add 1 cc. of Molybdate I and 0.4 cc. of aminonaphtholsulfonic acid, dilute to the mark, and use after 15 minutes. A photoelectric colorimeter with an orange filter may be used. In this case the appropriate aliquot portion of the bone solution is placed in a glass-stoppered 10 cc. volumetric flask and the procedure described for the standard is followed.

Calculation—By visual colorimeter.

Mg. P in aliquot

$$= \frac{\text{reading of standard}}{\text{reading of unknown}} \times \frac{\text{volume of unknown}}{\text{volume of standard}} \times \text{amount of P in standard}$$

$$\text{Mg. P in bone} = \text{mg. P in aliquot} \times \frac{\text{total volume of bone solution}}{\text{volume employed in analysis}}$$

The results obtained by this method were checked by the analysis of known amounts of phosphorus and also by the gravimetric method of Washburn and Shear on femurs from the opposite side of the animal. The data are presented in Table II and show close correspondence for the two methods.

Neither precipitation, turbidity, off color solutions, nor other difficulties were encountered with the above procedure in contrast to other variations attempted.

Determination of Total Base

The electro dialysis method of Keys (13), modified by Sobel, Yuska, and Cohen (14), is used. The modified construction of the apparatus (Fig. 2) and the technique described have been used with good results in this

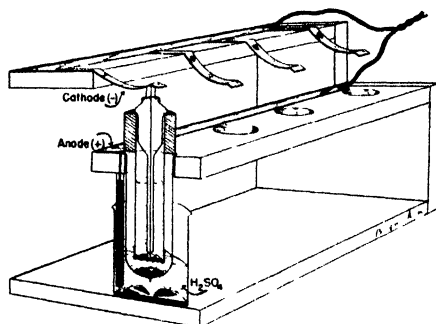


FIG. 2. Construction of electro dialysis apparatus used for total base estimation

laboratory for a number of years. In this modification boric acid is employed to trap the cations and Ma and Zuazaga's indicator is used.

Apparatus—The modified design of the apparatus is shown in Fig. 2. The cathode electrodes are obtainable from the Macalaster-Bicknell Company, Cambridge, Massachusetts.

Procedure—Details of the apparatus are given in Fig. 2. 5 cc. of doubly distilled water are placed in the outer chamber and 2 cc. of 2 per cent boric acid in the inner cathode chamber. The apparatus is assembled, the circuit is closed, and electro dialysis proceeds for 1 hour. The current is then stopped, the acid in the inner chamber aspirated off, and the mercury and the chamber are washed with several portions of doubly distilled water. The blank is thus eliminated by a preliminary electro dialysis (15). 2 cc. of 2 per cent boric acid are again placed in the inner chamber. A volume of bone solution containing about 0.02 milliequivalent of total base is pipetted into the purified, doubly distilled water in the outer chamber. It is important that a sufficient excess of acid is present in the inner chamber to

trap all the cations. Electrodialysis is started through a 500 ohm resistance for the first 20 minutes and continued for 3 hours at full current. The current is stopped, the cathode electrode removed and rinsed with 1 cc. of doubly distilled water, and 1 drop of Ma and Zuazaga's indicator is added to the inner chamber which is removed and titrated with 0.0100 N HCl to match the color of 2 cc. of boric acid with the indicator diluted to the same volume.

Calculation—

M.eq. total base in bone

$$= \frac{\text{cc. acid} \times \text{normality of acid} \times \text{total volume of bone solution}}{\text{volume of bone solution used for analysis}}$$

The values for total base agree with those reported in the literature, ranging up to about 5 per cent in excess of the calcium present. (Data will be presented elsewhere.) In five sets of duplicate determinations on electrodialed calcium standards the following amounts of calcium were recovered (0.500 mg. present): 0.500, 0.500; 0.502, 0.500; 0.496, 0.494; 0.498, 0.498; 0.500, 0.500.

Correction to Total Base for Ammonia

Ammonia present in the HCl solution, either preformed in the bone or formed by hydrolysis of organic matter in the HCl treatment, is measured with the total base by electrodialysis. In order to estimate the fixed base the ammonia was determined and the amount subtracted from the dialyzed bases.

The ammonia was determined by a submicro modification in routine use in this laboratory for the estimation of urea by aeration based on the principle of Van Slyke and Cullen (16), with boric acid to trap ammonia as employed by Sobel, Yuska, and Cohen (14). Pyrex test-tubes, with side arm, of 10 cc. capacity are used, fitted with Pyrex tubing (5 mm. outside diameter, 3 mm. bore, and 18 cm. long) which will pass through a No. 0 soft rubber stopper. 2 cc. of 2 per cent boric acid-indicator solution are placed in the rear tube. 5 cc. of a mixture of 7 parts of 0.1 per cent alcoholic brom-cresol green + 1 part of 0.1 per cent alcoholic methyl red are added to every 100 cc. of 2 per cent boric acid solution. 0.1 cc. of bone solution is transferred to the front tube + 3 drops of caprylic alcohol, and 1.0 cc. of half saturated K_2CO_3 is added to liberate the NH_3 from solution; the apparatus is aerated and the ammonia trapped in boric acid and titrated with 0.0714 N HCl with a capillary micro burette.

No relation was found between the amount of ammonia and total base present.

SUMMARY

A scheme of analysis for bone and other calcified material is described which is serial in operation.

This scheme requires only one weighed specimen for analysis of CO_2 , Ca, P, and total base.

A complete analysis can be easily performed on as little as 5 mg. of sample.

Material in any form or shape can be accommodated for analysis.

We wish to acknowledge the assistance of Mr. Jerome Weinstein in the early phases of the investigation.

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THE DISTRIBUTION OF CHOLINESTERASE IN THE BOVINE RETINA

By CHRISTIAN B. ANFINSEN

(From the Department of Biological Chemistry, Harvard Medical School, Boston)

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It is recognized from the embryological development of the retina that this tissue is a phylogenetic derivative of the brain. One may visualize the retina as composed of a succession of layers of tissue of varying morphological composition. The structures constituting these layers are typical examples of similar structures in the brain itself and include a receptor system, the rods and cones, in addition to cell bodies, transmitting fibers, and synapses. Thus, the results obtained from a study of the distribution of substances concerned with metabolic processes in the layers of the retina are of potential value for two reasons. First, the results may be considered in relation to the physiological functions of the retina as the organ of vision. Second, results obtained on isolated portions of the nervous structures of the retina may give information of more general application to other parts of the central nervous system not available for such precise separation and study.

That chemical substances liberated at autonomic nerve endings may act as mediators of the nervous impulse is supported by a large body of evidence. However, in the case of neuromuscular junctions, ganglionic synapses, and synapses in the central nervous system, conduction occurs at such a rate that, for the production of discrete impulses within the short periods of time required, the chemical mediator must be formed, released, and removed from the site of action with extreme rapidity. If cholinesterase is the specific agent by which the mediator, acetylcholine, is removed, it is probably present in high concentrations at localized points in order that it may effectively destroy this substance during the required time.

Since 1937, evidence has accumulated favoring the localization of the enzyme. This evidence has been adequately reviewed and need not be discussed here (1, 2). Such studies, hitherto, have been primarily concerned with the neuromuscular junction and the ganglionic synapse. Although Nachmansohn has determined the gross distribution of cholinesterase in the central nervous system, histochemical studies directly relating structure and concentration of cholinesterase in mammalian nervous tissue have not been performed.

The retina is one of the few tissues of the mammalian organism in which synaptic structures are to be found in a reasonably isolated and compact

form. It has been possible, in the present studies, to obtain sections of retina rich in synaptic material and to compare their cholinesterase activity with the activity of sections containing few or no synaptic structures.

Methods

The technical details involved in the preparation of horizontal retinal sections and in the identification of the layers present have been presented in a previous paper (3).¹ The sections were weighed after treatment with xylene and staining. The weighings were made on the quartz torsion balance described by Lowry (4).

The present studies were carried out exclusively with bovine retinas. The results reported for the receptor layer, therefore, represent pure rod material.

Since it is difficult to prepare retinal sections which are entirely homogeneous with regard to cytological structures present, camera lucida drawings were made of the sections for which this was not possible. The weight of any layer present in a section could then be calculated from the total weight of the section and from the relative area, determined by weighing paper cut-outs of the camera lucida projections. In the calculation of these layer weights, the assumption was made that all layers were of the same lipid-free, dry weight density.

The micro titrimetric method of Glick (5) was used for the determination of cholinesterase activity. This method depends upon an incubation of the tissue section with acetylcholine and veronal buffer with subsequent back titration with HCl to determine the extent of acetate liberation. The quantities of reagents recommended by Glick were slightly increased. Thus, the tissue sections were extracted with 10.6 c.mm. of 30 per cent glycerol, to which were added, after the required extraction period, 13.6 c.mm. of the substrate-buffer solution. After incubation for a suitable length of time at 38°, usually 2 hours, the reaction was stopped by the addition of 75.2 c.mm. of eserine-brom-thymol blue solution. The titration was carried out to pH 6.3 with 0.05 N HCl, a Rehberg burette with a total capacity of 100 c.mm. being used. Fresh substrate-buffer and eserine solutions were prepared for each experiment.

The total control titer due to buffer and tissue was determined in the following manner. Sections of tissue were isolated from a second sample of the retina used for cholinesterase determinations. These sections were

¹ Since the publication of this paper, an additional precaution has been introduced to prevent the dislodgment of the retina from the liver base during sectioning. A warm knife edge is used to melt together the edges of the retina sample and the liver base. The entire object holder and its attached tissue are then again thoroughly chilled in the freezing mixture.

carried through the same incubation procedure as were those sections used in the enzyme determinations, but with the glycerol drop containing the tissue and the substrate drop kept separate until the addition of the eserine solution.

Table I illustrates that these titrations are constant within experimental error (0.1 to 0.2 c.mm.) although the histological composition and weights of the sections vary considerably. The enzyme activities reported in the following section, therefore, were obtained by subtracting the amount of 0.05 N HCl required for the titration of the vessels containing tissue and

TABLE I
Examples of Control Titrations

Eye No.	Section No.	Layers present	Total weight	Titration in 0.05 N HCl
			γ	c.mm.
1	1	Outer nuclear, outer reticular	36.5	5.74
	2	Outer reticular, inner nuclear	43.3	6.08
	3	Outer reticular	16.2	6.10
	4	Ganglion	11.7	5.80
Average titer.....				5.93
" deviation.....				0.18
2	1	Rods, outer nuclear, outer reticular	15.9	4.96
	2	Rods, outer nuclear, outer reticular	24.4	4.72
	3	Inner nuclear, inner reticular	10.0	4.82
	4	Outer reticular, inner nuclear, inner reticular	9.2	4.92
Average titer.....				4.86
" deviation.....				0.09

substrate in the same drop from the average value of the control titer determined as described above.

Results

The distribution of cholinesterase in the layers of the retina has been studied in two types of experiments, the procedure depending on the cytological homogeneity of the retinal sections. In the first type, the preparation of camera lucida drawings was omitted. This simplification was possible owing to the isolation by dissection of samples relatively homogeneous in structure. However, in the majority of the experiments, the complex histological structure of the sections made the preparation of camera lucida drawings essential. The results of all the experiments of

both types led to the conclusion that the cholinesterase activity of the retina was concentrated in the synaptic layers.

The data will be largely presented in the form of graphs. However, to indicate clearly the experimental procedure and the implications of the results, a typical experiment in which sections were studied chemically after camera lucida drawings had been made is presented in Table II which summarizes both the histological composition and the cholinesterase activity of the sections obtained in this experiment. Sections used for the

TABLE II
Distribution of Cholinesterase Activity in Bovine Retina

All tissue weights are expressed in micrograms. For the average control titration see Table I, 4.86 ± 0.09 c.mm. of 0.05 N HCl.

Section No.	Layers present	Relative weight	Total weight	Per cent of area	Layer weight	Titer in 0.05 N HCl	Cholinesterase activity
						c.mm.	
1	Rods	287	17.4	78	13.6	4.22	0.64
	Outer nuclear	81		22	3.8		
2	Rods	317	30.7	55	16.8	3.82	1.04
	Outer nuclear	185		32	9.8		
	“ synaptic	77		13	4.0		
3	“ nuclear	29		13	1.6		
	“ synaptic	180	12.2	80	9.8	3.88	0.98
	Inner nuclear	14		7	0.8		
4	Outer synaptic	54	9.0	21	1.9	3.14	1.72
	Inner nuclear	75		29	2.6		
	“ synaptic	124		49	4.4		
5	Outer synaptic	6	15.1	1		1.78	3.08
	Inner nuclear	6		1			
	“ synaptic	318		76	11.5		
	Ganglion	88		22	3.2		
6	Nerve fibers		20.2	100	20.2	4.80	0.06

determination of the control titer were obtained from a separate sample of the retina from the same eye. The data for these latter sections have already been presented in Table I (Eye 2).

As has been described previously (3), sectioning proceeded from the surface of the retina-choroid sample nearest the rod layer, each section being 20μ in thickness. The sections used in the determination of cholinesterase activity were successive ones except in the case of Section 6 which was 80μ rather than 20μ distant from Section 5. The control sections were also cut consecutively at 20μ intervals.

As a result of the sectioning technique, the initial sections in the series were composed, predominantly, of the rod and outer nuclear layers. Thus,

Section 1 in Table II contains 78 per cent of rods and 22 per cent of outer nuclear layer. The calculated weights of these two components are found in the sixth column and were calculated, as previously described, from the total weight of the section and the relative areas of each layer present. It will be seen that as sectioning proceeded, the histological composition of the sections changed markedly. For example, in Section 5 the rods and

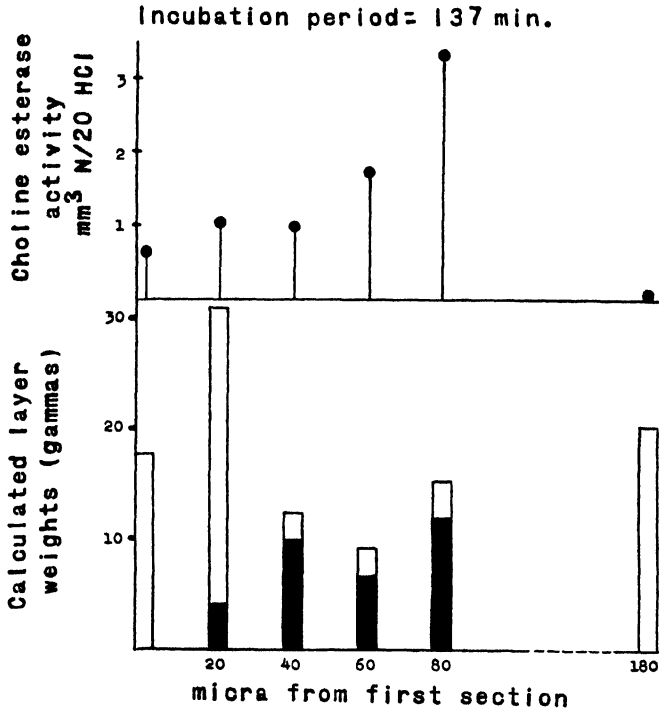


FIG. 1. The distribution of cholinesterase in the bovine retina. The shaded areas in the lower section indicate the calculated weights of total synaptic tissue. The unshaded portions represent non-synaptic tissue.

outer nuclear layer have disappeared completely, the major component of this section being the inner synaptic layer which comprises 76 per cent of the total weight of the section.

The relative activities of Sections 1 to 6 expressed in terms of c.mm. of 0.05 N HCl were obtained by subtracting the individual values determined from the average control titration. The highest activity is found in Section 5. Upon inspection of the data in Table II, it will be observed that this section, of all those tested in this experiment, contains the highest content of synaptic tissue. To make more apparent the relationship be-

tween cholinesterase activity and section composition, the results of the above experiment are plotted in Fig. 1. Two other experiments of the same technical nature are presented, graphically, in Figs. 2 and 3 which include both histological and chemical data. In the upper portion of Figs. 1 to 3, the enzyme activity has been plotted against the corresponding distance in micra of each section analyzed from the first section in the series. The lower portions summarize the weights of the various sections tested

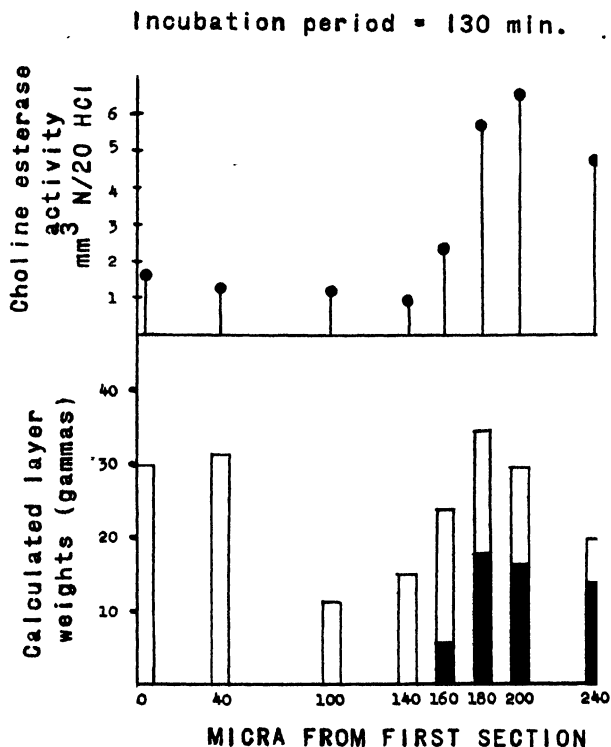


FIG. 2. The distribution of cholinesterase in the bovine retina. See Fig. 1 for the explanation.

enzymatically as well as the relative amounts of synaptic and non-synaptic tissue present in each section. Thus, in Fig. 1, the total height of the second column in the lower portion of the figure represents the total weight of Section 2 in Table II. The total height has been divided into two portions, the shaded portion, comprising 13 per cent, representing the weight of synaptic material in the section, and the remaining 87 per cent, the non-synaptic tissue. This method of representation is followed in each of Figs. 1, 2, and 3.

It is apparent from Figs. 1 to 3 that, as the proportion of synaptic tissue in the sections increases, the cholinesterase activity follows a similar course. Such a correlation cannot be made for any of the other layers in the sections.

If the cholinesterase activity is strictly proportional to the weight of synaptic tissue in the sections, a straight line relationship should be obtained upon plotting one against the other. Such plots of the data from Figs. 1, 2, and 3 are presented in Figs. 4, 5, and 6, respectively. It will be

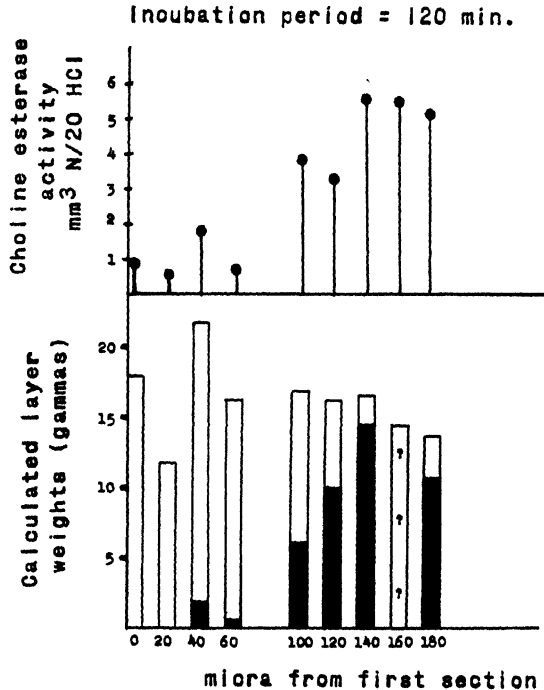


FIG. 3. The distribution of cholinesterase in the bovine retina. See Fig. 1 for the explanation.

observed that the hypothesis is satisfied by all of the experiments with the exception of one aberrant point in Fig. 4 (see also Fig. 1 from which the datum for this point is taken). To plot the results in this form, the assumption must be made that the activities of the inner and outer synaptic layers are identical, since the abscissae represent the *sum* of the weights of both of these layers in any section. This assumption seems to be permissible, since a number of the points on the plots represent sections which contain only one or the other of these layers.

Further experiments on the cholinesterase activity of some of the layers have been performed on samples of the outer nuclear, rod, inner synaptic, and outer synaptic layers, dissected essentially free of other contaminating

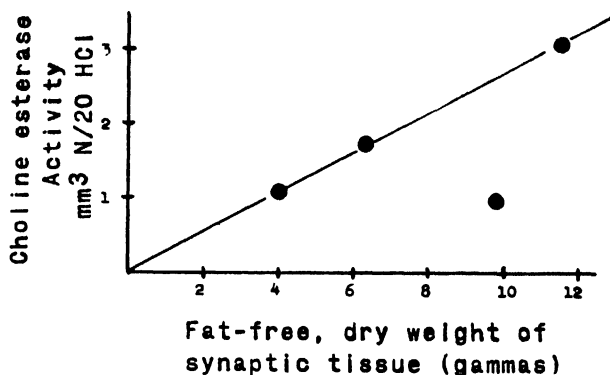


FIG. 4. Relation of cholinesterase activity to the synaptic content of retinal sections. The data are taken from Fig. 1.

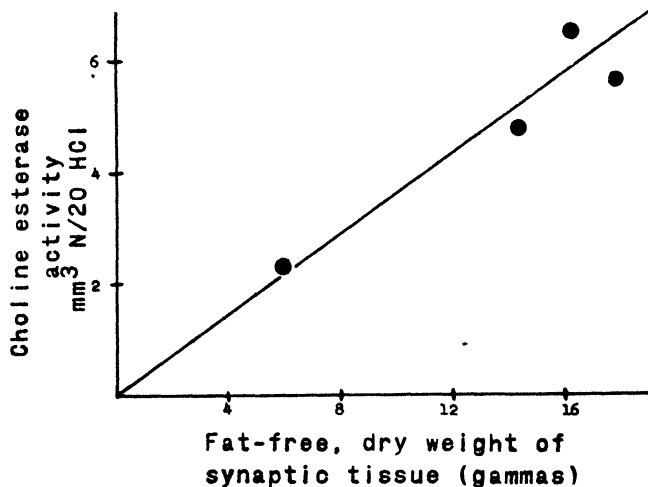


FIG. 5. Relation of cholinesterase activity to the synaptic content of retinal sections. The data are taken from Fig. 2.

layers. It will be seen in Table III that the activity of samples of the rod and outer nuclear layers is uniformly low. The results of similar studies on the synaptic layers of the retina are found in Table IV. These results are plotted in Fig. 7 to test the correlation between synaptic weight and

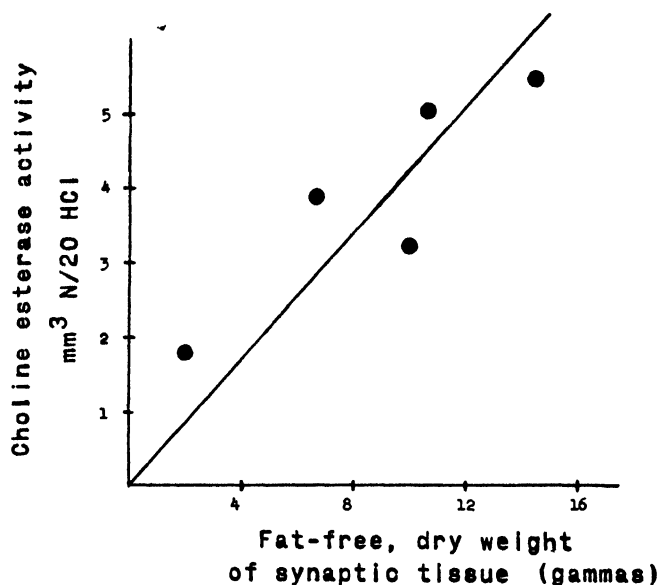


FIG. 6. Relation of cholinesterase activity to the synaptic content of retinal sections. The data are taken from Fig. 3.

TABLE III
Cholinesterase Activity of Rod and Outer Nuclear Layers of Retina

Eye No.	Sample No.	Section No.	Layers present	Weight	Per cent of area	Cholinesterase activity*
1	I	1	Rods	22.4	100	-0.08
		2	"	33.9	90	0.06
		3	Outer nuclear	40.0	40	-0.16
			Rods	34.5	60	
		4	" "	73.8	100	0.05
1	II	1	Rods	73.8	100	0.16
2	I	1	"	30.6		0.12
			Outer nuclear			

* Cholinesterase activity is expressed in terms of c.mm. of 0.05 N HCl per hour and represents the difference between the titer of each vessel and the average control titer.

enzyme activity. The straight line relationship indicates, again, the predominantly synaptic localization of cholinesterase activity.

In the bovine retina, the inner nuclear layer is too thin to permit the

isolation of cytologically homogeneous sections with the present techniques. For this reason, no direct data are available on the relative cholinesterase activity of this layer. Only occasionally have sections of the ganglion and

TABLE IV
Cholinesterase Activity of Outer and Inner Synaptic Layers of Retina of Eye 1

Sample No.	Section No.	Layers present	Per cent of area	Weight	Weight of layers	Cholinesterase activity*
I	1	Inner synaptic	100	γ	γ	
II	1	Outer nuclear	90	10.4	10.4	1.22
		" synaptic	10	25.2	22.7	0.39
	2	" "	80	25.7	20.6	2.44
		Inner nuclear	20		5.1	
I	2	Ganglion	50	25.5	12.3	0.16
		Nerve fiber	50		12.3	

* Cholinesterase activity is expressed in terms of c.mm. of 0.05 N HCl per hour.

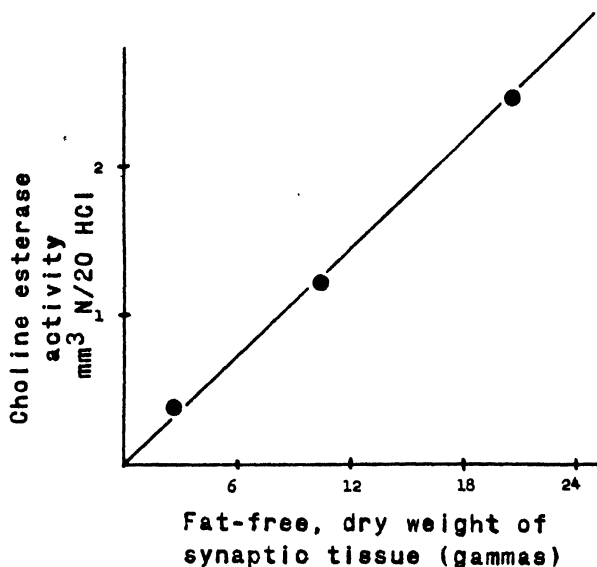


FIG. 7. Relation of cholinesterase activity to the synaptic content of retinal sections. The data are taken from Table IV.

nerve fiber layers been obtained owing to the damage to this portion of the retina which often occurs during the mounting and sectioning procedures. However, the last point in Fig. 1, at 180 μ , is an example of such a section

containing only nerve fibers and showing very low cholinesterase activity. Table III includes data for a section containing equal amounts of ganglion cells and nerve fibers. Here again, very low activity was observed.

DISCUSSION

The studies of Nachmansohn and his collaborators (1, 6) and of Glick (7) have yielded strong, though indirect, evidence that the enzyme cholinesterase exists in high concentrations at localized positions in the nervous system. In these studies, certain regions known to be high in synaptic endings and motor end-plates have been analyzed for their cholinesterase activity. However, in no studies of the mammalian nervous system have histological composition and cholinesterase activity been quantitatively related. Thus, conclusions regarding the importance of chemical mediation across the synaptic barrier, though highly probable, have hitherto rested on inferential evidence.

In the present studies, the histological structure of retina, with its cytologically homogeneous layers, has permitted the isolation and study of essentially pure synaptic material and of adjacent non-synaptic material. Thus, it has been possible to study the cytological localization of cholinesterase in a portion of the nervous system by a quantitative and direct method of analysis. The graphs (Figs. 4 to 7) relating synaptic weight to cholinesterase activity lend support to the hypothesis that the enzyme is, for the most part, concentrated at the synapse.

Boell and Nachmansohn (8) and Fulton and Nachmansohn (9) have suggested that cholinesterase is situated on the surface of all conducting nervous structures and that the amounts to be found along the nerve fibers are related to the concentrations at the synapse in accordance with the respective surface areas of these portions of the conducting path. Although it was not possible to determine the relative surface areas of the nerve fibers, cells, and synapses in the retina with present histological techniques, the results presented herein are not inconsistent with Nachmansohn's proposal.

Although marked localization of cholinesterase in the synapses of the retina has been found in the present studies, it is not possible to account completely for synaptic transmission in terms of the acetylcholine-cholinesterase system until quantitative knowledge of two other factors is available. These are (a) the amount of acetylcholine requiring hydrolysis, and (b) the volume to which the acetylcholine and cholinesterase, *in vivo*, are confined. It has been shown in several instances (1, 7) that there exists sufficient enzyme to satisfy the requirements of the theory, but only when a certain minimum localization of enzyme and substrate is assumed. Final decision as to the degree to which the acetylcholine system satisfies

the physiological requirements for transmission of nerve impulses in the body must, therefore, await further study.

SUMMARY

1. By employing single horizontal sections of the retina both for quantitative histological analysis and for chemical study, it has been possible to determine the localization of the enzyme, cholinesterase, in the layers of this organ. The results indicate a predominantly synaptic localization of cholinesterase. This provides direct support for the current theory of chemical mediation of the nervous impulse.

2. The usefulness of the retina as a test organ for the study of the general physiology of otherwise inaccessible components of the central nervous system has been pointed out.

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THE DISTRIBUTION OF DIPHOSPHOPYRIDINE NUCLEOTIDE. IN THE BOVINE RETINA

By CHRISTIAN B. ANFINSEN

(From the Department of Biological Chemistry, Harvard Medical School, Boston)

(Received for publication, October 13, 1943)

The presence of diphosphopyridine nucleotide (DPN) has been demonstrated in most mammalian tissues. However, the distribution of this important coenzyme among the various types of cells present in individual tissues has not been studied.

It is well known that the retina possesses extremely high rates of respiration and glycolysis. In view of the fact that DPN is an essential component of the oxidative and glycolytic systems and since it is found in the retina in greater amounts than in any of the other body tissues (Sym, Nillson, and von Euler (11)), it was felt that a study of the distribution of this substance in the layers of the retina, made up of different cell types, would be of particular interest. By such studies, the relative importance of DPN-linked reactions in structurally different parts of the retina would be indicated.

In the following pages are reported the results of experiments on the determination of the DPN content of isolated layers of the retina. As Table I will indicate, large variations were found in the values for different portions of the retina, the highest values being obtained for the synaptic regions. The implications of these findings will be discussed with particular reference to the transmission of the visual impulse.

Methods

With the previously described histological techniques (Anfinsen, Lowry, and Hastings (3) and Anfinsen (1)), samples of most of the retinal layers, essentially homogeneous with respect to cytological composition, were isolated by dissection of frozen dried sections under xylene. The tissue samples were weighed on a quartz torsion balance (Lowry (7)) and subjected to analysis by the micro modification (Anfinsen (2)) of the method of Jandorf, Klemperer, and Hastings (6) for the determination of DPN.

It has not been possible to obtain samples of the inner nuclear layer owing to its thinness in the bovine retina. Values for the nerve fiber layer have also been omitted, since this layer is generally damaged during the mounting and sectioning procedures.

The results have been expressed in terms of micrograms of DPN per microgram of lipid-free tissue solids. Owing to the lack of information concerning the water and lipid content of the various layers, it is at present impossible to convert these values to a fresh tissue basis. However, the use of the former weight unit is an advantage rather than a disadvantage, since the fat-free dry weight of tissue gives a more accurate estimate of the actively metabolizing, cellular phase.

For the microdetermination of DPN, fresh muscle extract was prepared routinely every 3 days from the same supply of muscle acetone powder. The activity of the extract prepared from this powder was tested in the standard arsenate system for the determination of DPN (Jandorf, Klemperer, and Hastings (6)). A value of 35 c.mm. of CO_2 per microgram of DPN per hour at 30° was obtained in these calibration experiments.

Results

The results obtained in the experiments are presented in Table I and are summarized graphically in Fig. 1. The values obtained for each layer were sufficiently similar to permit averaging of the data. As will be seen in Table I, it was frequently possible to obtain a number of samples of a single layer from one retina.

Layer of Rods—The layer of rods was found to contain an average of 2.3×10^{-3} γ of DPN per microgram of fat-free, dry weight of tissue. Two sections (Table I) contained 40 and 50 per cent, respectively, of pigment epithelium. Since no further analyses are available on this layer owing to its extreme thinness (about 12μ), little more can be said than that the DPN content of this layer of pigment epithelium appears to be approximately the same as that of the rod layer.

Outer Nuclear Layer—An average DPN content of 1.7×10^{-3} γ per microgram of fat-free, dry weight of tissue was obtained for this layer. In one eye, No. 5, considerably lower values were obtained. In view of the agreement of the values on two separate samples of the retina from this eye, the variation is probably a real, biological one. It will be seen in Table I that analyses performed on samples of the inner synaptic layer from Eye 5 are also considerably different from the rest of the determinations. It seems likely that this particular eye represents an isolated case.

Outer Reticular Layer—The average DPN content of this layer was 4.0×10^{-3} γ per microgram of fat-free, dry tissue. No significant variations were observed from eye to eye.

Inner Reticular Layer—As was mentioned above, only one analysis differed seriously from the average DPN content of 4.1×10^{-3} γ per microgram of tissue. This single value has not been included in the average.

It will be seen, upon comparison with the other layers studied, that the

TABLE I
DPN Content of Layers of Retina

Layer of cells	Eye No.	Weight of tissue	DPN $\times 10^3$	DPN $\times 10^3$ per microgram fat-free, dry weight	Average	Average deviation
		γ	γ	γ	γ	γ
Rods	3	17.2	41.8	2.4	2.3×10^{-3}	0.2×10^{-3}
	3	13.0	38.1	2.9		
	4	18.9	35.0	1.9		
	4	25.1	60.2	2.4		
	4	18.7	37.4	2.0		
	4	11.4	24.3	2.1		
	5	20.7	57.2	2.7		
	7	10.3	23.6	2.3		
	7	10.3*	22.2	2.2		
	8	12.3†	28.5	2.3		
Outer nuclear	3	14.3	29.6	2.1	1.7×10^{-3}	0.3×10^{-3}
	3	9.9	25.6	2.1		
	4	19.8	33.4	1.7		
	6	29.7	32.1	1.8		
	8	24.6	41.2	1.6		
	5	33.0	32.5	1.0		
	5	28.1	33.3	1.2		
Outer reticular	1	14.6	63.3	4.3	4.0×10^{-3}	0.3×10^{-3}
	7	15.0	61.8	4.1		
	8	19.4	71.3	3.7		
	8	20.8	71.4	3.5		
	8	13.7	50.0	3.7		
Inner reticular	8	16.2	71.8	4.4	4.1×10^{-3}	0.2×10^{-3}
	1	16.8	64.6	3.9		
	3	11.2	46.4	4.1		
	4	19.2	85.3	4.4		
	4	24.2	105.0	4.3		
	4	16.3	60.8	3.8		
	5	11.2	69.0	6.1‡		
	6	14.4	54.9	3.8		
Ganglion	8	15.9	68.0	4.3	3.7×10^{-3}	0.2×10^{-3}
	1	8.4	31.0	3.7		
	6	10.2	33.5	3.5		
	6	13.8	58.9	4.2		
	6	16.0	58.9	3.7		
	8	12.9	40.0	3.2		
	8	9.0	33.4	3.7		

* The section contained pigment epithelium comprising approximately 40 per cent of the total area. The relative area was estimated by inspection of the stained section under the microscope.

† This section contained approximately 50 per cent pigment epithelium.

‡ Not included in the average (see the text).

two synaptic regions contain the highest amounts of DPN of any of the retinal zones. Indeed, the values are the highest yet reported for any tissue; rat liver contains approximately 3.5×10^{-3} γ per microgram of fat-free, dry tissue (Jandorf (5)).

Ganglion Cell Layer—The analyses indicate a DPN content of 3.7×10^{-3} γ per microgram of tissue, a value approximately equal to that reported for rat liver.

Nerve Fiber Layer—As was pointed out above, data on this layer could not be obtained for technical reasons. In Fig. 1, it has been assumed

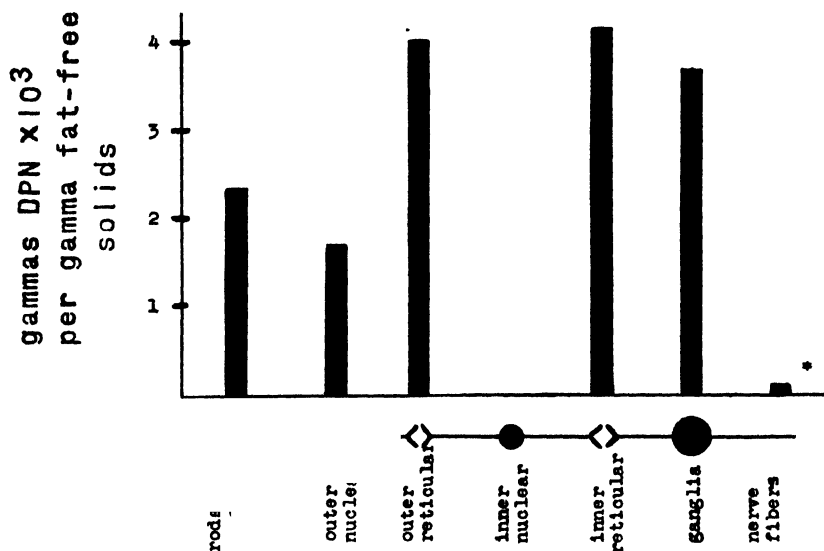


FIG. 1. The distribution of diphosphopyridine nucleotide in the bovine retina

* Calculated for nerve fiber tissue from the data of Sym, Nillson, and von Euler (11).

that the value found for the DPN content of nerve fibers in general (Sym, Nillson, and von Euler (11)) is of the same order of magnitude as that of the nerve fibers in the retina.

DISCUSSION

It is well known that DPN is a ubiquitous component of protoplasm and, in combination with specific enzymes, serves to carry out certain dehydrogenations. It is not known at present whether there is any direct relation between the concentration of DPN in protoplasm and its metabolic activity. However, it is reasonable to assume that, under certain conditions in which DPN-linked reactions limit the metabolic rate of a cell, the higher the concentration of DPN, the greater the number of DPN-linked dehydrogenations which can occur in unit time. The results reported

herein and graphically presented in Fig. 1 indicate that, whereas the nerve fibers are poor in DPN, the ganglion cells and synapses are rich in DPN. The concentration of DPN in the rods and outer nuclear layer is about half that of the synaptic material.

The high values obtained for the two synaptic regions are of particular interest in view of certain recent findings on the mode of formation of acetylcholine and its rôle in the chemical mediation of the nervous impulse across the synapse. It has been shown (Anfinsen (1)) that in the retina, cholinesterase is also markedly concentrated in the synaptic regions. Such a finding, in accordance with the modern concepts of chemically mediated synaptic transmission (Fulton and Nachmansohn (4); Nachmansohn (9)), indicates the possibility of cholinergic mediation of the visual impulse. Quastel and his associates (8, 10) have shown that the formation of acetylcholine from pyruvate and choline is an oxidative process. It is probable that oxidation-reduction coenzymes, among them DPN, play a rôle in this series of reactions. Therefore, in view of the highly specialized function of the synapse and of the high concentrations of DPN found in these regions of the retina, the hypothesis is presented that transmission of the nerve impulse across the retinal synapse may be dependent upon one or more DPN-linked reactions.

Although the involvement of DPN in the oxidative acetylation of choline by pyruvate has not been demonstrated, it is known that the formation of pyruvate from carbohydrate, the main metabolite of retinal tissue, requires the presence of DPN. Thus, the high concentrations of DPN in the reticular layers of the retina may be concerned with both the formation of pyruvate and its utilization in these layers to form acetylcholine.

SUMMARY

Quantitative histochemical studies of frozen dried, retinal sections indicate that the concentration of diphosphopyridine nucleotide varies considerably from layer to layer in this organ. The highest concentrations were found in the two synaptic regions. The layer of ganglion cells also contained considerable quantities of the coenzyme. The rods and outer nuclear layer, however, were found to contain much smaller amounts.

The results have been discussed with regard to the possible rôle of DPN in the transmission of the visual impulse through the mediation of acetylcholine.

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A MICRO MANOMETRIC METHOD FOR THE DETERMINATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE

By CHRISTIAN B. ANFINSEN

(From the Department of Biological Chemistry, Harvard Medical School, Boston)

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In the course of histochemical studies of the retina, it was desired to determine the diphosphopyridine nucleotide content of single microtome sections of tissue weighing on the order of 10 to 50 γ . It was, therefore, necessary to increase the sensitivity of the manometric method for the determination of DPN. To this end, the technique of Linderstrøm-Lang (6) with the Cartesian diver was employed.

Of the manometric methods available, the method of Jandorf, Klemperer, and Hastings (4) was chosen for the present purposes owing to the availability and stability of the components of the test system and to the relatively large gas evolution per microgram of DPN. By the micromethod described below 0.001 to 0.006 γ of DPN can be determined with an error of less than 5 per cent. This represents a thousandfold increase in sensitivity of the method. Briefly, the principle of the method is as follows: In the presence of arsenate and an extract of acetone powder of cat muscle, the dismutation of triose phosphate to phosphoglyceric acid and phosphoglycerol, normally coupled with the phosphorylation of adenylic acid or adenosine diphosphate, occurs without simultaneous phosphorylation. The dearsenylation of 1-arseno-3-phosphoglyceric acid, an intermediate in the over-all reaction, proceeds spontaneously. The molecule of acid formed in this step liberates carbon dioxide from the bicarbonate buffer. When DPN is the limiting factor in the system, the rate of gas production is strictly proportional to the concentration of the coenzyme.

Details of Micro Technique

Reagents and Enzyme Solution—The concentration of reagents (sodium bicarbonate, sodium arsenate, and sodium hexose diphosphate solutions) recommended by Jandorf *et al.* were used in all experiments. These were combined to form the stock solution (designated herein as "substrate-buffer" solution) and stored in the refrigerator. The enzyme solution was prepared by the usual method from an acetone powder of cat skeletal muscle. However, for the removal of interfering nucleotides from the muscle preparation, it was found that the 20 hour charcoal adsorption period could be greatly reduced. Thus, the initial adsorption was allowed to proceed for 1 hour followed, after centrifugation, by a second adsorption of the super-

natant with a fresh portion of charcoal for $1\frac{1}{2}$ hours. The DPN blank for such a solution is always small immediately after adsorption and disappears completely after storage for 12 to 24 hours at 6° .

Manometric Apparatus Employed—The rate of gas evolution was measured with the apparatus of Linderstrøm-Lang (6) employing the principle of the Cartesian diver. The apparatus was of standard design and permitted the successive measurement of five divers in any one experiment. The water bath was maintained at $30^{\circ} \pm 0.01^{\circ}$. Brodie's solution was used in the arms of the manometer. As a flotation medium for the divers, a solution was prepared containing 27.2 gm. of NaNO_3 , 13.7 gm. of NaCl , and 59.1 gm. of water. This medium has as high a molarity as the saturated LiCl solution usually employed in the diver apparatus and, in addition, has a much lower viscosity. This property permits greater control of the diver's movements during readings. Its density, 1.321, was frequently checked with a hydrometer.

Steps Involved in Filling Divers—When the divers are filled with the various components of the test system, it is essential that all final dilutions correspond with those used in the macro technique. Thus, in the original procedure, 1.3 cc. of the substrate-buffer solution are diluted in the Warburg vessels to a final volume of 3.0 cc., representing a dilution of 2.3. For use in the divers, this dilution is brought about as follows: To 1 part of the substrate-buffer solution is added 0.17 part of a DPN solution, either of known concentration for calibration experiments, or resulting from tissue extraction. After thorough mixing, a 1.60 c.mm. aliquot is pipetted into the diver with a pipette of the type illustrated in Fig. 1, *F*. The total volume is then made up to 3.14 c.mm. with water and muscle extract. The over-all dilution of the substrate-buffer solution now agrees with that employed in the macro technique, being $(1.17/1.00) \times (3.14/1.60) = 2.3$. As long as the correct proportionality in concentration is observed, the volume taken may be varied according to the needs of the experiments and the availability of micro pipettes.

To make the volume in the diver up to the correct total with muscle extract and water the same type of procedure is followed, the muscle extract and water being combined into one solution of the correct dilution and pipetted into the divers in one operation. In our experiments, 1 part of muscle extract was diluted with 0.49 part of distilled water. 1.54 c.mm. of this solution were then pipetted into the diver bulb. The dilution is, therefore, $(1.49/1.00) \times (3.14/1.54) = 3.0$, corresponding to the dilution of muscle extract employed in the macro technique.

The diver is now flushed with a gas mixture composed of 5 per cent CO_2 :95 per cent N_2 for 3 minutes to bring the pH to 7.4. The gas is led into the diver through a capillary tube of small diameter. The tip is inserted into the diver to within a mm. of the surface of the reaction mixture.

As rapidly as possible, the neck of the diver is closed with a ring of paraffin oil. This oil must previously have been thoroughly saturated with the same gas mixture as that used above. The oil ring is introduced with

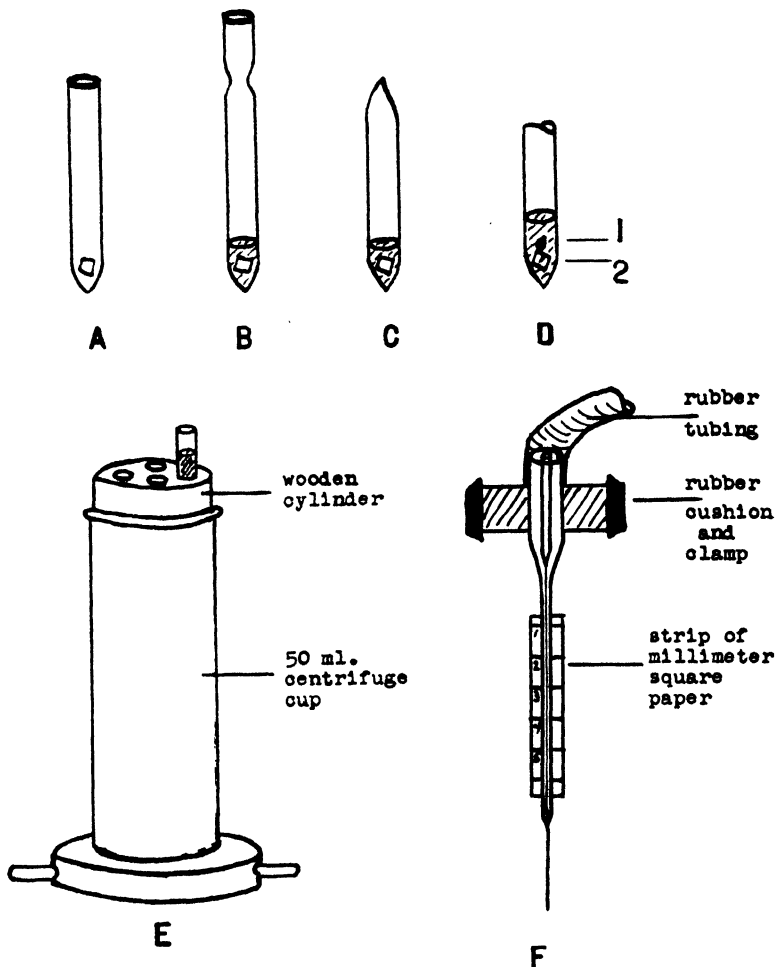


FIG. 1. Apparatus used in the extraction of diphosphopyridine nucleotide from frozen dried sections. *A*, micro centrifuge tube with frozen dried section; *B*, tube after addition of water; *C*, tube sealed off during heat inactivation; *D*, tube during extraction of DPN.

the same type of pipette used for the aqueous solutions. The pipette rack and rack and pinion device for raising and lowering the divers during filling were of the type described by Linderstrøm-Lang and Holter (8).

The diver is transferred to the flotation tube and is allowed to come to

temperature and pressure equilibrium over a period of 5 minutes. Readings taken before this time has elapsed are not reliable.

When the required number of divers have been filled and equilibrated, readings are begun. A control diver is included as a thermobarometer. This diver contains the same solutions as the experimental divers with the exception of the DPN solution which is replaced by water. To obtain reliable curves, the readings are made at 5 minute intervals.

The c.mm. of CO_2 produced during 1 hour are computed by multiplying the change in equilibrium pressure during this period (obtained graphically

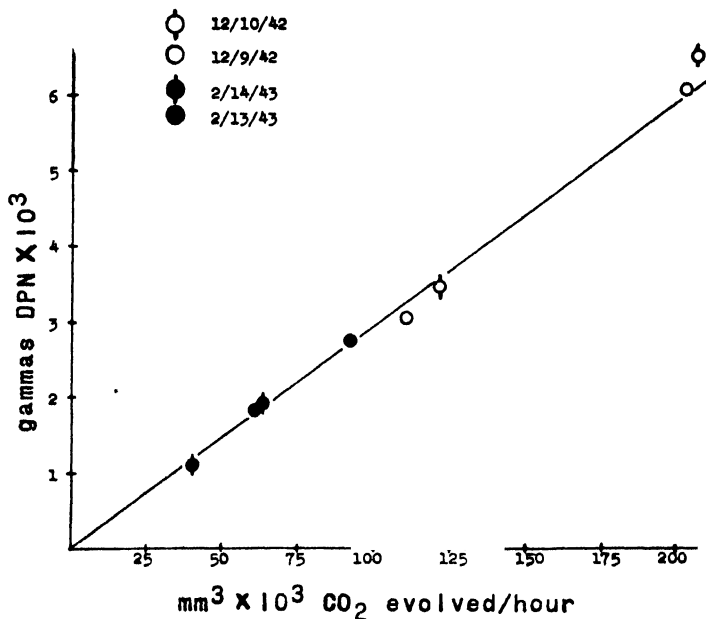


FIG. 2. Calibration of the arsenate system at 30° by technique of the Cartesian diver.

from a plot of equilibrium pressure *versus* time) by the diver constant. This constant is calculated as follows:

$$k = \frac{V \times \frac{273}{T} + V_L \alpha_L + V_o \alpha_o}{10,000}$$

where V , V_L , and V_o are the volumes of the gas phase in the filled diver, volume of aqueous solution, and oil volumes, respectively, and α_L and α_o the solubility of CO_2 in water and oil at 30°. These latter constants have

the values of 0.66 and 1.03, respectively (Dixon (2); International Critical Tables).

The total volume of the vessel is determined by subtracting the weight of the diver from the weight when filled with water. The gaseous phase in the neck of the diver occasionally protrudes into the flotation medium, forming a small bubble. However, the volume of such a gas bubble causes an error in the value of the constant of the order of 1 per cent, and for this reason is disregarded.

Calibration of Micro Test System—The micro technique and original method with the Warburg apparatus were compared by running parallel calibration curves on solutions of the same DPN sample (prepared by Dr. B. J. Jandorf). This sample was found to contain 40 per cent DPN by spectrophotometric analysis.

The results of four calibration experiments with the Cartesian diver are presented in Fig. 2. The slope of the curve, 35 c.mm. of CO₂ per hour per microgram of DPN, duplicates, within experimental error, the values obtained with the Warburg apparatus.

Results obtained at 38° by both methods were also in agreement. However, unless the demands of the problem require that determinations be made at 38°, it is recommended that 30° be chosen as the working temperature for the micromethod, as described above. The enzyme solution is more stable at this temperature and, in addition, complications introduced by the higher temperature during the manometric readings and filling the diver are avoided.

Extraction of DPN from Frozen Dried Sections

Jandorf (3) has shown that in intact portions of excised rat liver the DPN content of the tissue slowly decreases with elapsed time after death, reaching a stable value at two-thirds of its initial value after about 20 minutes. For the histochemical studies of the retina to which the microdetermination of DPN was applied, frozen dried sections of tissue have been used. Although it will be seen from experiments to follow that freezing and subsequent dehydration prevent the rapid disappearance of DPN, this disappearance does take place as soon as the dried tissue is once again exposed to an aqueous medium. In order to preclude such disappearance in frozen dried sections of liver and in other tissues, hitherto untested, which also might exhibit this phenomenon, it is important to inactivate rapidly the destructive enzyme systems as soon as the extracting medium is added. With the small amounts of material used for the present studies, the evaporation of water upon heat inactivation at 100° causes considerable error in the calculation of dilutions. Therefore, a method was devised to circumvent this difficulty, some apparatus for which is shown in Fig. 1.

The stained sections (see Anfinsen, Lowry, and Hastings (1)) were weighed on a quartz torsion balance (Lowry (9)) and transferred to micro centrifuge tubes (A) which were then drawn out to form a narrow, thin walled constriction as in (B). These tubes were generally prepared from 4 mm. Pyrex tubing, but occasionally, when the weight of tissue to be analyzed required smaller total amounts of extract, 3 mm. tubing was used.

With a constriction pipette of the design described by Levy (5), a known amount of distilled water was added, and the tube was rapidly sealed by drawing out the narrow portion in a small flame (C). The enzymes were immediately inactivated by plunging the sealed tube into boiling water.

TABLE I

DPN Analyses of Rat Liver by Micro Manometric Method

Inactivation of both fresh and frozen dried tissue was carried out 590 seconds after death. To convert the dry weights of the sections to a fresh tissue basis, the weights were divided by the factor 0.29 (Manery and Hastings (10)).

	Section 1	Section 2	Section 3	Section 4	Section 5	Macro-determination
Date of experiment*	Jan. 9	Jan. 14	Jan. 14	Jan. 14	Jan. 14	Jan. 5
Dry weight, γ	104.4	67.7	84.3	53.3	103.3	
Fresh weight, mg	0.360	0.233	0.298	0.183	0.355	
C.mm. CO ₂ per section per hr.....	5.40	3.60	4.20	2.75	5.16	
Micrograms DPN per section†.....	0.154	0.106	0.127	0.081	0.152	
Micrograms DPN per gm. liver.....	427	454	426	443	428	425

Average DPN content, 435 γ per gm.; average deviation, 10 γ per gm.

* The frozen dried sections were prepared on January 5, 1943, at which time the DPN content of the fresh liver was determined in duplicate.

† 1 γ of DPN produces 35 c.mm. of CO₂ per hour in the arsenate system at 30°.

After 2 minutes, the tube was removed and thoroughly chilled in ice-cold water. Drops of solution adhering to the sides and top were then centrifuged to the bottom in a special centrifuge cup (E) which was designed to hold four such tubes. The tube was then opened with a sharp file as in (D). A measured amount of substrate-buffer solution was added and thorough stirring carried out by means of the electromagnetically operated "flea" (Linderstrøm-Lang and Holter (7)) (D-1) until the section (D-2) had been well extracted. After another centrifugation, the tube was stoppered and stored at 6° until needed for the chemical determination. The relative amounts of water and substrate-buffer solution employed in the extraction must be chosen so that the final dilution in the divers corresponds

to the concentrations of the components of the test system recommended by Jandorf *et al.* (4). It is also essential that the total volume yield a dilution of DPN which will be satisfactory for use in the Cartesian divers.

In parallel experiments, the DPN content of rat liver was determined by the standard macro technique of Jandorf *et al.* and by the micromethod described above. The sample taken for analysis in the Warburg apparatus was heat-inactivated (see Jandorf (3)) at the same time that the tissue to be used for preparing frozen dried sections was plunged into petroleum ether and dry ice. If the freezing and extraction procedures adequately prevent DPN disappearance, the DPN content of these portions of liver should be identical. That this identity was obtained will be seen by comparing the results of the micro and macro experiments in Table I. Table I also furnishes evidence for the stability of DPN in frozen dried sections when stored in stoppered vessels at 6°. It will be observed that over a period of 9 days, under these conditions, no decrease in the DPN content of the sections occurred.

SUMMARY

A micro manometric method for the determination of the diphosphopyridine nucleotide of very small amounts of tissue has been described. By this method 0.001 to 0.006 γ of DPN can be determined with an error of less than 5 per cent.

The extraction of DPN from frozen dried sections has been discussed and analyses carried out on the extracts are shown to agree with the values obtained with the standard Warburg apparatus.

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A SIMPLE QUARTZ TORSION BALANCE

By OLIVER H. LOWRY

(From the Department of Biological Chemistry, Harvard Medical School, Boston)

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In connection with histochemical studies of the retina (1, 2) a need was encountered for a balance to weigh frozen dried tissue sections having a mass of 5 to 50 γ . A precision of $\pm 0.1 \gamma$ was desired.

A satisfactory balance (Fig. 1) was obtained with the principle employed in a balance described by Barrett, Best, and Ridout (3). The beam (4) consists of a hollow quartz tube 25 cm. in length and about 1 mm. in diameter. This tube is suspended in a quartz yoke (B) between two horizontal quartz fibers (C).

The pans (D) which are made of aluminum foil are suspended from quartz hooks (E) which in turn are suspended from fine quartz loops in the ends of the beam. No attempt was made to make the two halves of the beam of exactly equal length.

Unequal loading of the beam causes a displacement which is opposed by the resultant torsion in the quartz fibers. The displacement of one end of the beam is measured with a cathetometer (F) reading to ± 0.01 mm.¹ This displacement is proportionate to the weight added to the pan. In the balance described a displacement of 1.00 mm. is equivalent to 10.8 γ .

The balance is mounted in an old balance case by sealing the feet (G) of the balance to the floor with de Khotinsky cement. The mechanism used for lifting the beam of the original balance is retained (not shown) and is provided with a stirrup to lift the quartz beam just enough to steady it when additions are made to the pans. In order to reduce the effects of static electricity, the inside of the balance case is covered with metal foil except for small windows (H) at each end to permit observation with the cathetometer. As a further precaution the balance is metalized with a thin film of platinum to make it conducting. To accomplish this, a 5 per cent solution of chloroplatinic acid in alcohol is allowed to flow along all the members of the balance and after drying is heated with a cool flame until conversion to platinum occurs. Great care must be taken not to overheat the fine quartz suspensions. After mounting, the balance is grounded to the case by means of a strip of aluminum foil. Readings are made with the cathetometer after the balance case has been closed for 1 minute. The cathetometer is focused on any convenient landmark on one

¹ We have used a cathetometer manufactured by the Gaertner Scientific Corporation, Chicago.

end of the beam. The end of the beam, which is in comparative darkness inside of the closed balance case, is easily visible against the white background of an illuminated piece of paper outside of the opposite end of the balance.

In order to calibrate the balance, a piece of fine wire 5 or 10 cm. in length and weighing 1.5 to 2 mg. is selected. This wire is cut into ten pieces of nearly equal length and all ten pieces are weighed together on an analytical micro balance. Each piece is then placed separately in turn on one pan and the displacements are observed. The sum of all the separate displacements divided by the known total weight gives the sensitivity. Since the two balance arms are not exactly equal, this will only calibrate one end of the balance.

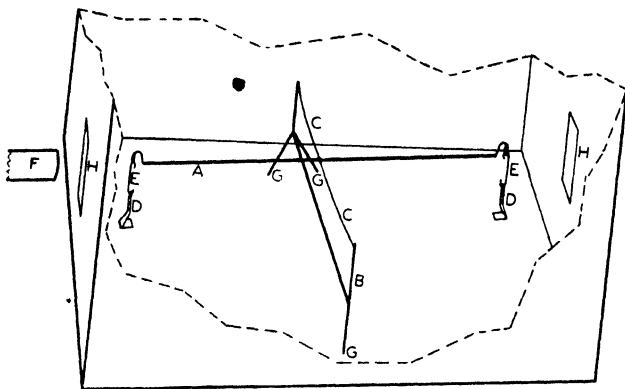


FIG. 1

It is possible to weigh larger masses by partially balancing with known weights in the opposite pan. In this case the ratio between the two balance arms would have to be determined by (1) observing the zero point, (2) putting approximately equal weights on each pan, and (3) reversing them as is sometimes practiced with the ordinary analytical balance.

The balance described has given satisfactory use for over a year with no change in sensitivity, and in spite of the fine quartz suspensions employed, it is not especially fragile. It has a capacity of 50 to 100 mg. and a sensitivity of $\pm 0.1 \gamma$. The readings are reproducible and the zero point is quite stable.

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THE EFFECT OF SULFONAMIDES ON THE CONVERSION IN VITRO OF INORGANIC IODIDE TO THYROXINE AND DIIODOTYROSINE BY THYROID TISSUE WITH RADIOACTIVE IODINE AS INDICATOR*

BY A. L. FRANKLIN AND I. L. CHAIKOFF

(From the Division of Physiology, University of California Medical School, Berkeley)

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MacKenzie *et al.* found that the administration of several of the sulfonamides produced a hypertrophy and hyperplasia of the thyroid gland (1). This observation was confirmed by Astwood *et al.* (2, 3). Although the evidence obtained by these workers makes it appear that these compounds diminish the production of the thyroid hormone, the exact site of action of the sulfonamides (whether in the gland or elsewhere), as well as the reactions affected, is not definitely known. According to Astwood *et al.*, there is no evidence against the view that the action of the compounds is elsewhere than in the gland and is "concerned with the synthesis of some essential component or precursor of the hormone" (2).

An examination of the sequence of reactions, beginning with the removal of inorganic iodide from the blood stream by the thyroid gland and ending with the release of the circulating hormone by the gland, shows that the sulfonamides could interfere with any of the following reactions, among others: (1) the selective removal of circulating inorganic iodide by the gland, (2) the incorporation of inorganic iodide into diiodotyrosine and thyroxine, (3) the release of the hormone into the blood stream. It is demonstrated here that the sulfonamides interfere directly with the conversion of inorganic iodide to diiodotyrosine and thyroxine. Evidence for this direct action of the sulfonamides on the iodine metabolism of the gland was obtained by measuring the incorporation of labeled inorganic iodine (I^{131}) into diiodotyrosine and thyroxine by surviving slices of thyroid tissue. It was previously established with the aid of the radioactive isotope of iodine that surviving preparations of thyroid tissue obtained from the rat, sheep, and dog readily convert inorganic iodide to thyroxine and diiodotyrosine (4).

EXPERIMENTAL

Thyroid slices were prepared from the sheep, cow, and dog. The animals were sacrificed by exsanguination. The glands were removed, immediately

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packed in ice, and kept there during the short interval that preceded their slicing.

Preparation of Bicarbonate-Ringer's Solution Containing Sulfanilamide—The preparation of the bicarbonate-Ringer's solution containing tracer amounts of radioactive iodide (I^{131}) has been described elsewhere (4). Concentrated solutions of sulfanilamide were made up in 0.9 per cent NaCl of such strength that the addition of 0.1 cc. of these solutions to a 3.0 cc. volume of the radioactive bicarbonate-Ringer's solution yielded the concentrations of sulfanilamide recorded in Table I.

TABLE I

Effect of Sulfanilamide on Formation of Diiodotyrosine and Thyroxine in Thyroid Slices

Experiment No.	Sulfanilamide concentration	Per cent of Ringer's I^{131} recovered as	
		Thyroxine	Diiodotyrosine
1. Beef	M		
	0 (Control)	10.4	34.5
	0 "	9.8	42.2
	10^{-3}	1.4	2.0
	10^{-2}	1.5	4.6
	10^{-3}	2.4	6.1
	10^{-3}	2.4	11.2
	10^{-4}	7.8	31.8
	10^{-4}	6.5	31.8
	10^{-5}	9.7	37.7
2. Sheep	10^{-5}	8.9	35.4
	0 (Control)	8.5	50.9
	0 "	8.3	56.3
	0 "	8.2	59.4
	10^{-3}	1.4	6.4
	10^{-3}	1.5	5.2
	10^{-3}	1.5	4.4

Preparation of Bicarbonate-Ringer's Solutions Containing Sulfapyridine, Sulfaguanidine, Sulfadiazine, and Sulfathiazole—Owing to their insolubility it was not possible to prepare concentrated solutions of the other sulfonamides in the manner described above. A large volume of bicarbonate-Ringer's solution containing the desired concentration of the sulfonamide was first prepared, and to a small aliquot of it was added the I^{131} contained in 0.9 per cent NaCl; the volume of the latter was small in comparison with the total volume of bicarbonate-Ringer's solution.

300 mg. of thyroid slices were transferred to 25 cc. Erlenmeyer flasks containing 3.0 or 3.1 cc. of the solutions described above. The bicarbonate-Ringer's solution was saturated with a gas mixture consisting of 5 per cent CO_2 and 95 per cent O_2 before it was added to the reaction flask, and the

atmosphere above the solution was displaced with this same gas mixture immediately after the addition of the slices. The flasks were then tightly stoppered and incubated at 38° for 2 hours. The vessels were gently agitated during the entire period of incubation.

In the experiments recorded in Tables I to III, the entire contents of each flask were analyzed for radiothyroxine and radiodiiodotyrosine by the procedure described elsewhere (4).

In the penetration experiments recorded in Table IV, the slices were rinsed twice as described below and the total radioactivity contained in the slices determined. The slices *only* were transferred to flasks containing 6.0 cc. of 2 N NaOH and heated on a steam bath till a clear solution was obtained. It was made up to volume and an aliquot used for determination of total radioactivity.

Results

Formation of Radiothyroxine and Radiodiiodotyrosine

Sulfanilamide—The results of two experiments with this sulfonamide are recorded in Table I. Experiment 1 was done with surviving slices of cow thyroid; Experiment 2, with slices of sheep gland. Four concentrations of sulfanilamide were tested. The inhibitory effects of 10^{-2} M and 10^{-3} M were quite marked. At lower concentrations, namely 10^{-4} M and 10^{-5} M, this compound no longer appreciably influenced the conversion *in vitro* of inorganic iodide to either thyroxine or diiodotyrosine.

Sulfapyridine—At a concentration of 10^{-3} M, sulfapyridine definitely interfered with the formation of radiothyroxine and radiodiiodotyrosine (Table II). No effect was observed when the thyroid slices were incubated in a Ringer's medium containing 10^{-5} M sulfapyridine. In Experiment 1 the radiothyroxine formed in the presence of 10^{-4} M sulfapyridine was reduced to one-half of that of the controls; the amount of radiodiiodotyrosine formed was also reduced in this experiment. In Experiment 2, the conversion of inorganic I^{131} to thyroxine was slightly reduced by a concentration of 10^{-4} M sulfapyridine, but the amount of radiodiiodotyrosine formed did not differ significantly from that of the controls.

Sulfaguanidine—Three concentrations (10^{-3} M, 10^{-4} M, and 10^{-5} M) of this sulfonamide were used (Experiments 3 and 4 of Table II). Definite inhibition was observed only with the highest concentration.

Sulfathiazole—A decreased formation of radiothyroxine and radiodiiodotyrosine was found at both concentrations tested; *viz.*, 10^{-3} M and 10^{-4} M (Table III).

Sulfadiazine—The concentrations used in the study of this compound were limited by its solubility. In Experiment 3 (Table III), no inhibition in the formation of radiothyroxine and radiodiiodotyrosine was observed

when the thyroid slices were incubated in a Ringer's medium containing 10^{-4} M sulfadiazine. In Experiment 4, however, a decreased formation of radiodiiodotyrosine was found in the presence of this concentration of sulfadiazine.

Penetration of Inorganic I^{131} into Thyroid Slice

The results obtained above show that the sulfonamides interfere with the conversion of the I^{131} of the medium to thyroxine and diiodotyrosine. In addition to converting inorganic iodide to thyroxine and diiodotyrosine,

TABLE II
Effect of Sulfapyridine and Sulfaguanidine on Formation of Diiodotyrosine and Thyroxine in Thyroid Slices

Experiment No.	Sulfapyridine concentration	Per cent of Ringer's I^{131} recovered as		Experiment No.	Sulfaguanidine concentration	Per cent of Ringer's I^{131} recovered as	
		Thyroxine	Diiodotyrosine			Thyroxine	Diiodotyrosine
	M				M		
1. Dog	0 (Control)	9.2	60.0	3. Dog	0 (Control)	9.5	57.9*
	0 "	9.7	55.8		10^{-3}	4.7	28.0
	10^{-3}	1.2	13.7		10^{-3}	4.7	29.4
	10^{-4}	5.6	42.7		10^{-4}	8.6	51.2
	10^{-4}	6.7	42.7		10^{-4}	8.2	58.1
	10^{-5}	10.4	61.0		10^{-5}	10.4	59.8
2. Sheep	0 (Control)	8.7	59.0	4. Sheep	10^{-5}	10.1	63.5
	0 "	8.6	57.0		0 (Control)	8.7	58.0†
	10^{-3}	5.2	36.2		10^{-3}	4.2	40.8
	10^{-3}	3.8	29.1		10^{-3}	4.5	36.2
	10^{-4}	6.9	61.5		10^{-4}	6.5	56.5
	10^{-4}	7.0	62.5		10^{-4}	6.9	58.7

* The control experiments are the same as those shown in Experiment 1. Only average values are therefore given here.

† Control experiments the same as in Experiment 2; only average values given here.

however, the thyroid gland has an extraordinary capacity for accumulating iodine. This is demonstrated in the experiments of Table IV, in which the iodine-concentrating capacities of thyroid slices are compared with those of liver slices.

In the experiments of Table IV the procedure was essentially the same as that used in the experiments recorded in Tables I to III. The slices were prepared in the usual manner and then transferred to reaction flasks containing 0 (i.e. controls) or 10^{-3} M sulfanilamide in addition to the radioactive iodide. At the end of a 2 hour period of incubation, the slices were sepa-

rated from the Ringer's medium. The radioactive Ringer's solution was decanted from each reaction flask and all the slices rapidly transferred to a Syracuse watch-glass containing 3 cc. of non-radioactive Ringer's solution.

TABLE III

Effect of Sulfathiazole and Sulfadiazine on Formation of Diiodotyrosine and Thyroxine in Sheep Thyroid Slices

Experiment No.	Sulfathiazole concentration	Per cent of Ringer's I^{131} recovered as		Experiment No.	Sulfadiazine concentration	Per cent of Ringer's I^{131} recovered as	
		Thyroxine	Diiodotyrosine			Thyroxine	Diiodotyrosine
1	M			3	M		
	0 (Control)	8.7	58.0*		0 (Control)	8.7	58.0*
	10^{-3}	2.3	7.1		10^{-4}	7.7	58.5
	10^{-3}	1.7	9.5		10^{-4}	7.9	60.2
	10^{-4}	4.3	35.7		0 (Control)		52.2†
2	10^{-4}	3.9	33.0	4	10^{-4}		19.2
	0 (Control)		50.1		10^{-4}		16.9
	0 "		61.1		10^{-4}		15.6
	0 "		45.5				
	10^{-3}		6.0				
	10^{-3}		4.1				
	10^{-3}		4.3				

* The control experiments are the same as those shown in Experiment 2, Table II; only average values given here.

† Control experiments the same as in Experiment 2 above; only average values given here.

TABLE IV

Effect of Sulfanilamide on Penetration of Inorganic Iodide (I^{131}) into Tissue Slices

Experiment No.	Tissue (sheep)	Sulfanilamide concentration	Per cent of Ringer's I^{131} recovered in slices
1	Thyroid	M	
	"	0 (Control)	79
	"	0 "	82
	"	10^{-3}	79
	"	10^{-3}	78
2	Liver	0	9
	"	0	10
	Thyroid	0 (Control)	88
	"	0 "	85
	"	10^{-3}	81
3	"	10^{-3}	78
	"	0 (Control)	97
	"	0 "	98
	"	10^{-3}	87

The slices were permitted to remain in this solution for 20 seconds and then transferred for 20 seconds to another 3 cc. volume of non-radioactive Ringer's solution. The slices were then removed and analyzed for total I^{131} . Since the washing in the two non-radioactive solutions served to remove I^{131} adhering to the surfaces of the slices, the I^{131} found in the slices was taken as a measure of the iodine-concentrating capacity of the tissue.

The capacity of the thyroid tissue to remove inorganic I^{131} from the Ringer's medium was not appreciably depressed by the presence of sulfanilamide in the reaction flask. The slight depression in iodine-concentrating capacity observed in Experiments 1, 2, and 3 (Table IV) was in no way comparable to the degree of inhibition of the conversion of inorganic I^{131} to thyroxine and diiodotyrosine (see Table I). Hence it is not the reduction in the iodine-concentrating capacity of the gland that is responsible for the failure of the conversion of the Ringer's I^{131} to thyroxine and diiodotyrosine in the presence of sulfanilamide.

DISCUSSION

At a concentration of 10^{-3} M, sulfanilamide, sulfapyridine, sulfathiazole, and sulfaguanidine depress the formation *in vitro* of thyroxine and diiodotyrosine by thyroid tissue. Although the mechanism by which these compounds produce this effect is still unknown, it should be noted that at a concentration of 10^{-3} M sulfanilamide did not interfere appreciably with the extraordinary capacity of thyroid tissue to absorb inorganic iodide from the surrounding medium. It is the subsequent conversion (*i.e.* after the inorganic iodide has entered the gland) to thyroxine and diiodotyrosine that is specifically inhibited by the sulfonamides.

Little can be said at present about the action of the sulfonamides on specific enzyme systems concerned with the formation of thyroxine and diiodotyrosine in the thyroid gland. The formation of these two iodinated compounds by this tissue has been shown to involve oxidations in which the cytochrome-cytochrome oxidase system participates (5). This enzyme system, however, is not affected by the sulfonamides, for at concentrations as high as 10^{-2} M Mann and Keilin found they had no effect on cytochrome oxidase (6). Lipmann has made the interesting observation that the catalytic oxidation of *p*-aminobenzoic acid by peroxidase is inhibited by the sulfonamides (7), but it is not yet known whether this reaction or other enzyme reactions affected by the sulfonamides are linked with the conversion of inorganic iodide to thyroxine and diiodotyrosine in the gland.

It was pointed out some time ago that it was not possible to say definitely whether the radioactive inorganic iodide added to the reaction flask reacts with iodinated molecules, such as diiodotyrosine and thyroxine, or with uniodinated molecules, such as tyrosine and thyronine, contained in the

surviving thyroid slices (4). The only evidence provided against the view that this is an interchange reaction was the finding that the incorporation *in vitro* of I^{131} into diiodotyrosine and thyroxine occurs only in the presence of intact surviving slices of thyroid tissue but fails to occur (a) when organization of the slice is disrupted by homogenization (4) and (b) in the presence of cytochrome oxidase inhibitors (5). The recent observations of MacKenzie and MacKenzie (1) and of Astwood *et al.* (2) indicating that the sulfonamides depress hormone formation by the thyroid gland, together with the present findings (*i.e.* that the sulfonamides inhibit the conversion *in vitro* of inorganic I^{131} to radiothyroxine and radiodiiodotyrosine by surviving thyroid slices), lend additional support to the view that the incorporation of inorganic I^{131} into thyroxine and diiodotyrosine by surviving thyroid slices as measured here is not an interchange reaction but represents the formation of new molecules of thyroxine and diiodotyrosine.

SUMMARY

1. The effects of sulfanilamide, sulfapyridine, sulfaguanidine, sulfathiazole, and sulfadiazine on the conversion *in vitro* of inorganic I^{131} to thyroxine and diiodotyrosine by surviving slices of thyroid tissue were determined.

2. At a concentration of 10^{-3} M, sulfanilamide, sulfapyridine, sulfaguanidine, and sulfathiazole inhibited the formation of radiodiiodotyrosine and radiothyroxine.

3. The iodine-concentrating capacity of surviving thyroid slices (*i.e.* their capacity to remove I^{131} from the Ringer's medium) was not appreciably depressed by the presence of 10^{-3} M sulfanilamide in the medium.

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COVITAMIN STUDIES

I. THE SPARING ACTION OF NATURAL TOCOPHEROL CONCENTRATES ON VITAMIN A*

By K. C. D. HICKMAN, MARIAN WOODSIDE KALEY, AND PHILIP L. HARRIS

(From the Laboratories of Distillation Products, Inc., Rochester, New York)

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The term "vitamin E" has been applied to α -tocopherol and to a lesser extent to the other tocopherols and certain synthetic compounds (1) which are variously able to restore fertility to the depleted rat and cure muscle dystrophy in the depleted rabbit. Recently, there has been recognized another activity of these substances; namely, the power to augment the A vitamins (2, 3). A search of the literature (4) and exploratory assays in our laboratory suggest that this activity is perhaps a general one, extending beyond the protection of vitamin A. It is typical of the second activity that the individuality of the modifying agent is not then in evidence; the agent merely influences the physiological activity of the other vitamin. The phenomenon, which has come to be known as the "sparing" or "synergistic" action of vitamin E, will be referred to in these articles as "covitamin E" activity. The words synergy and synergistic will be retained for convenience although it is recognized that they should be reserved, strictly speaking, for those instances in which a stoichiometric or a specific physiological linkage is involved. Covitamin E activity is not specific and is shared interchangeably by the E vitamins and their congeners and even quite unrelated substances.

The present series of papers will embrace a wide field, in an attempt to cover covitamin E activity with the A vitamins, vitamins C and D, and certain hormones. Through a dietary survey coupled with developments in analysis designed to exclude substances in food hitherto mistaken by us for vitamins E, an attempt will be made to correlate laboratory findings with human needs.

The experimental methods follow a simple pattern. Conditions are sought under which the covitamins are substantially absent and the vitamin under study is present in border line amounts. Groups of animals in this standardized condition are then given increasing quantities of the covitamin over a wide, logarithmically spaced range and the relative performances of the groups noted. An example will illustrate one way of using the method: 80 depleted rats are each given 0.5 γ of crystalline vitamin A daily and are assembled into eight groups of ten animals, one group receiving no vitamin

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E, the others increasing quantities of tocopherols as follows: 0.015, 0.05, 0.15, 0.5, 1.5, 5.0, and 15.0 mg. daily. The average weight gain of each group is noted over a 3 to 5 week period.

The necessity of exploring the synergy over a wide dosage range has been encountered often in our work and through correspondence with other laboratories where there has been failure to observe the phenomenon. Sparing action is an interrelation between quantities of two or more accessory substances and must, therefore, be examined by a quantitative procedure not hitherto often required in vitamin assays. It is our experience that varying the quantity of a covitamin can provide an assay result below, equal to, or above that of the vitamin in a covitamin-free diet. Only a graduated series of assays can demonstrate the existence, position, and magnitude of the synergy. Another cause of failure encountered in our early work is the accidental use of a diet deficient in more than the two vitamins under study. With a deficiency of vitamin B complex brought about by a faulty batch of yeast, the proportion of the vitamin E needed was altered.

It may be asked whether a synergy which can be observed only under such narrow and rigidly controlled conditions can possibly be of importance in practical nutrition. The answer, we feel, is definitely affirmative. In the laboratory, where the diet must contain plentiful supplies of all the food factors except the one under study, border line conditions are hard to secure. In poorer homes and in war-torn countries, border line conditions are the rule rather than the exception. Similar conditions arise on the farm. The cow, for instance, in a natural habitat has enjoyed such a bountiful supply of carotene that an efficiency of absorption of 0.1 to 1.0 per cent has amply sufficed for its vitamin A needs. The same animal domesticated and living on hay and dried alfalfa for half the year is on the nutritional border line for vitamin A (5). Indeed, relieving this condition may well provide a test case for an applied vitamin A-E synergy.

The effect of synthetic α -tocopherol in increasing the storage of vitamin A in the liver of the rat was discovered by Moore and associates (6, 7). Their classic experiments were both the starting point and inspiration of this research. Our laboratory had for some years been convinced that the major rôle of vitamin E in nutrition would be found linked with its antioxidant rather than its antisterility powers. Moore's experiments and, concurrently with our own first attempts, those of Sherman (8) and Quackenbush (2) pointed the way to a general experimental technique.

Moore's experiment was with liver storage of vitamin A. Whether increased storage was accompanied with lessened or enhanced availability of this vitamin A for the rat was not revealed. We have recast the experiment to test this point by attempting to answer the following questions.

Do varying quantities of vitamin E (1) alter the vitamin A depletion time of weanling rats; (2) alter the survival time on a vitamin A-free diet of rats previously receiving a large supplement of vitamin A; (3) alter the response of depleted rats to minimal doses of vitamin A; (4) affect the standard U.S.P. vitamin A assay?

EXPERIMENTAL

A first requisite is a diet sufficiently low in vitamin A to fail to support life, and sufficiently low in vitamin E to respond to very small supplements of that substance. It was less important for the diet to be completely deficient in vitamins A and E than that all other essential dietary factors should be retained unspoiled. For this reason, preparatory steps which involved natural rancidification, treatment with FeCl_3 , etc., were avoided. Most of the vitamin E in the standard U.S.P. diet for vitamin A depletion is

TABLE I
Composition of Diets

	Diet 10	Diet 18
Casein (purified), %.....	18	18
Salt Mixture 2 (U.S.P. XII), %.....	4	4
Starch, %.....	35	35
Cerelose, %.....	30	30
Dried brewers' yeast (tested sample), %.....	8	8
Vitamin D ₃ , units per gm. diet.....	5	5
" B ₁ , γ per gm. diet.....	10	10
Wesson oil (approximately 0.04% tocopherol), %.....	5	
Olive " (no tocopherol), %.....		5

contained in the specified 5 per cent of vegetable oil, usually cottonseed or corn (Wesson or Mazola). The U.S.P. diet for assay of vitamin A prepared with olive oil and fortified with vitamin B₁ provided a suitable ration for our experiments and one which was particularly sensitive to variations of vitamin A or E supplements. In order to assure ourselves that extra vitamin E may also be of benefit to the rat on a diet containing an amount of this vitamin, by common consent considered sufficient, we retained the U.S.P. diet unchanged in some experiments. This diet with Wesson oil is referred to as Diet 10, with the olive oil-substituted ration as Diet 18. Their composition is given in Table I.

The vitamin A supplements were, variously, the crystalline alcohol, crystalline acetate, concentrates molecularly distilled from shark and dogfish liver oil, and U.S.P. Reference Oil II. The vitamin E supplements were a concentrate from the molecular distillation of soy bean and cottonseed oil

products. The concentrate contained 40 per cent of an equal mixture of natural α - and γ -tocopherols with small, unknown quantities of the β compound.¹ The concentrate, which remained remarkably constant in composition from one batch to another, was chosen because it represented the most plentiful source of the vitamin, and the findings, if useful, could at once be put to practical account. We shall show in a later paper that the sparing action is shared in equal degree by the three tocopherols, with the mixture, perhaps because of overlapping oxidation-reduction potentials, appearing to possess enhanced activity.

Both vitamin A and E concentrates were given by calibrated dropper. If the vitamins were dissolved in the olive oil and incorporated in the diet, the vitamin A was completely lost, perhaps by surface oxidation during the hours between mixing and consumption. If the vitamin A was given by dropper and the vitamin E in the olive oil-feed mixture, the synergistic

TABLE II
Effect of Tocopherols on Vitamin A Depletion

Tocopherol supplement	No. of rats	Time for depletion	Standard error	Significance (P)*	Gain in weight during depletion	Standard error	Significance (P)*
mg. per day		days	days	per cent	gm.	gm.	per cent
0	27 M.	30.5	0.78	3	71.6	2.1	0
0.3	25 "	33.6	1.14		83.1	3.4	
0	10 F.	32.3	1.28	13	65.2	3.6	90
0.3	10 "	35.9	1.77		64.4	5.6	

* These values represent the chances that the differences concerned may be accidental. Those less than 5 per cent are considered significant.

effect was absent or at least far inferior to that produced by an equivalent quantity of vitamin E as naturally occurring in the Wesson oil of Diet 10. Extra vitamin E added to the Wesson oil and then to the diet failed to confer commensurate benefit with vitamin E administered by dropper either on Diet 10 or 18. In so far, then, as our findings can be projected to human nutrition or that of farm animals, the synergy would be expected when the vitamin E is administered by capsule or bolus, and not expected when added as a concentrate to prepared foods.²

Vitamin E and Vitamin A Depletion Period—Approximately 70 rats were

¹ The tocopherol concentration in per cent was calculated by multiplying the value of *E* (1 per cent, 1 cm.) (295 m μ) by the factor 1.25.

² The failure of vitamin E concentrates to survive in powdered food should be regarded as a temporary situation which is being overcome as various details of technique are mastered.

placed on Diet 18, *ad libitum*, from the time of weaning. One-half of the group was given 0.3 mg. of tocopherols in olive oil by dropper. The depletion time and weight gains are recorded in Table II. The difference between the mean gain in weight for the male animals receiving tocopherol and those receiving none is highly significant. The difference in depletion period is also statistically significant. Owing to the smallness of the groups of female animals, the slight differences between them has no significance as an isolated experiment. However, in all the data accumulated a lesser sensitivity to vitamin E and a lesser mortality on border line supplements are seen to have been exhibited by young female rats compared with males.

Survival Time Following Single Large Dose of Vitamin A—Five groups of depleted rats were maintained on Diet 18 and the number of days noted for

TABLE III

Effect of Tocopherols on Response of Depleted Rats to Single Dose of Vitamin A (Eight Male Rats to a Group)

Tocopherol supplement	Vitamin A supplement	Growth response					Average survival time
		4 days	12 days	20 days	28 days	36 days	
mg. per day	units per day	gm.	gm.	gm.	gm.	gm.	days
0	0	1.6	-5.7	-15.0	-31.0 (4)*		29 ± 2.5
1.5	0	-6.4	-10.3	-15.4	-20.9 (2)		39 ± 4.0
0	50	10.9	26.2	24.1	20.5	9.0	46 ± 3.1
0.15	50	8.6	20.4	25.9	21.0	15.8	52 ± 2.5
1.5	50	4.2	15.4	21.6	23.5	23.0	51 ± 1.7

* The figures in parentheses represent the number of rats that died during the experiment.

the survival of each animal. Animals in three of the groups received a single dose of vitamin A (distilled ester concentrate, 50 U.S.P. units) at the start; the others received no vitamin A. One of the control and one of the groups given vitamin A received no tocopherol, the others 0.15 mg. and 1.5 mg. daily, respectively, as recorded in Table III. In this and two other similar experiments a relatively small increase in weight gain and time of survival is provided by the tocopherols. It is evident from later work that the optimum tocopherol supplement lies between the two values chosen.

Effect of Vitamin E on Growth Induced by Daily Supplements of Vitamin A—Weanling rats were depleted on Diet 10 and then assembled in large groups which were given various forms of vitamin A daily. The large groups were split into subgroups and given quantities of tocopherols ranging

from 0.025 to 5.0 mg. daily. Diet 10 was used throughout the test period except where stated otherwise. Table IV shows an exploratory assay covering a relatively small range, which, nevertheless, demonstrates the

TABLE IV

Growth Response and Survival Time, of Groups of Eight Rats, Induced by Pure Vitamin A (0.48 γ per Day for 28 Days) and Influenced by Various Levels of Tocopherol (Diet 18)

Group No.	Tocopherol supplement	Growth response		Survival time	
		28 days	48 days	Average	Standard error
	mg. per day	gm.	gm.	days	days
I	0	8.6 $\pm$ 3.6	7.25 $\pm$ 1.4	43.5	5.8
II	0.05	20.7 $\pm$ 3.1	20.4 $\pm$ 2.8	67.8	3.2
III	0.15	28.8 $\pm$ 1.1	30.5 $\pm$ 3.7	53.4	3.6
IV	0.5	27.6 $\pm$ 3.5	29.3 $\pm$ 3.9	63.5	1.8
V	1.5	31.7 $\pm$ 3.5	39.0 $\pm$ 6.9	60.9	1.3

TABLE V

Growth Response Induced by Pure Vitamin A and Pure Vitamin A Acetate As Influenced by Tocopherol Supplementation

Mixed tocopherol supplements daily	Diet 18 + 0.57 γ vitamin A OH						Diet 18 + 0.40 γ vitamin A acetate					
	No. of rats*	Gain in			Stand-ard error, 35 days	Relative rate of growth†	No. of rats*	Gain in			Stand-ard error, 35 days	Relative rate of growth†
		16 days	28 days	35 days				16 days	28 days	35 days		
		gm.	gm.	gm.				gm.	gm.	gm.		
0.0	10	19.3	29.4	29.4	3.2	0.21	7	19.7	22.8	35.1	3.3	0.12
0.025	10	20.6	29.3	32.6	3.1	0.23	10	21.7	33.3	40.1	4.7	0.29
0.05	9	30.3	45.1	48.3	6.5	0.23	8	19.7	32.8	37.1	3.1	0.31
0.15	10	28.4	41.6	49.7	5.5	0.26	9	23.1	44.1	51.7	3.9	0.38
0.30	10	31.5	48.5	55.2	6.2	0.27	9	21.8	35.7	42.0	2.9	0.32
0.50	9	28.7	50.5	54.7	4.0	0.31	8	22.9	39.2	47.1	6.1	0.34
1.50	10	28.4	43.3	44.6	5.6	0.22	8	19.9	33.2	42.5	4.6	0.36
5.00	10	30.5	44.4	48.5	4.4	0.24	8	24.5	40.0	46.9	3.4	0.32

* Ten rats were used for each group. The number reported shows those that survived the experiment. Most of the deaths occurred in the first few days and indicated overdepleted animals.

† Calculated approximately as $\frac{\text{weight at 35 days} - \text{weight at 16 days}}{\text{weight at 35 days} + \text{weight at 16 days}}$

synergy excellently. A growth of 8.6 gm. in 28 days for the group receiving no tocopherol may be compared with a growth of 32 gm. for the group given the optimum amount and an estimated 24 gm. for an amount of tocopherol

equivalent to that in Diet 10, the standard U.S.P. assay diet, all in the 28 day period. At the end of 28 days, the vitamin A supplements were stopped, while the vitamin E was continued. The additional time that the rats survived was 43 days (average) in the case of vitamin A alone, 61 days with vitamin A and optimum vitamin E.

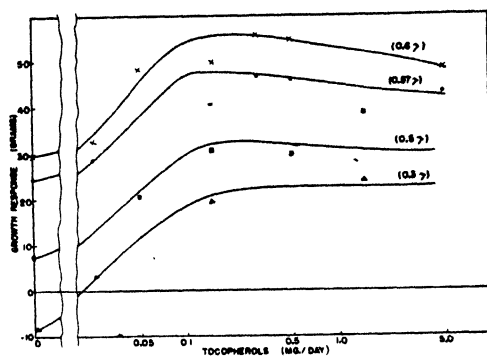


FIG. 1. Effect of tocopherols on growth response induced by vitamin A. Values in parentheses represent the amount of vitamin A fed daily during the 35 day experimental period.

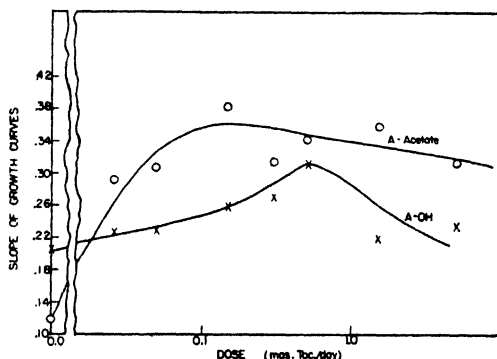


FIG. 2. Effect of varying tocopherol intakes on relative rates of growth response to vitamin A (see Table V).

More thorough surveys were made, with a slightly larger supplement of crystalline vitamin A and a smaller supplement of crystalline vitamin A acetate. The enhancing effect of vitamin E is evident with both compounds (Table V), reaching a maximum at about 0.15 mg. daily with the acetate and at about double the quantity with the free vitamin. This roughly parallels the relative quantities of tocopherol needed to provide equal stabilization of the two forms in oil solution *in vitro*.

The optimum position of the synergy varies slightly with the criteria

adopted, whether total gain in weight or maintenance of gain during the second half of the test period, reported under "slope of growth curve." The smaller dose of vitamin A acetate is seen to take a longer time to accumulate to the point of promoting growth than the larger dose of vitamin A, but its superior stability *in vivo* makes the total quantity available to the animals relatively greater as the assay proceeds. The relation between gain in weight and vitamin E supplement is shown in Fig. 1, and the

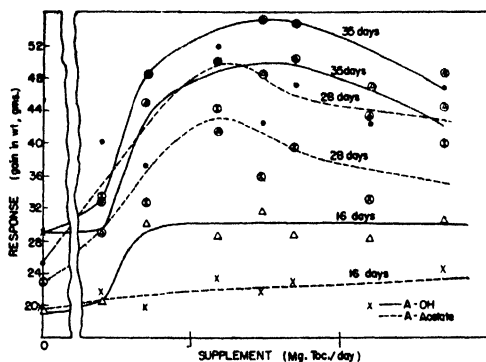


FIG. 3. Influence of tocopherols on growth induced by vitamin A at various times during the experimental period.

TABLE VI

Effect of Tocopherols on Utilization of Vitamin A from U. S. P. Reference Oil II

Mixed tocopherol supplements daily	Diet 10 + 1.4 units vitamin A daily		Diet 18 + 1.74 units vitamin A daily	
	No. of rats	Gain in 28 days	No. of rats	Gain in 28 days
mg.		gm.		gm.
0.0	11 (2)*	28.4	9 (2)	39.7
0.05	11 (3)	29.1	10 (2)	55.7
0.15	10 (0)	56.3	10 (0)	52.0
0.50	10 (3)	42.9	10 (0)	44.9
1.5	12 (3)	47.8	10 (0)	53.9
5.0	11 (4)	34.3	11 (2)	50.5

* The figures in parentheses refer to rats that died during the experiment.

relation between the slope of the growth curve and vitamin E in Fig. 2. Data from the experiment reported here and others are summarized in Fig. 3.

For the experiments with fish liver oil in place of pure vitamin A preparations U.S.P. Reference Oil II was used. The series was carried out in duplicate with the standard U.S.P. diet (No. 10) as well as Diet 18 to find out whether the vitamin A-E synergy should be taken into account in

routine assay procedures. The findings are recorded in Table VI. The ordinary U.S.P. diet is highly sensitive to the addition of tocopherols. It is interesting to note that the uniformity of response and general condition of the rats are better on Diet 18 with supplemented olive oil than on the more usual formula.

A number of questions arise from the last experiment, answers for which will be attempted in a later paper. Is the vitamin E synergy self-compensatory in standard assays, or does it affect the test sample and the reference oil in different degree? Does the synergy persist at higher dosage levels? The subject will be developed further in connection with carotene and vitamin E.

SUMMARY

Natural vitamin E (mixed tocopherols) is shown to enhance the growth-promoting power of vitamin A alcohol, vitamin A acetate, and U.S.P. reference oil.

The time taken to deplete young rats of vitamin A and the survival time after vitamin A supplementation has ceased are shown to be increased by vitamin E.

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COVITAMIN STUDIES

II. THE SPARING ACTION OF NATURAL TOCOPHEROL CONCENTRATES ON CAROTENE*

By PHILIP L. HARRIS, MARIAN WOODSIDE KALEY, AND K. C. D. HICKMAN

(From the Laboratories of Distillation Products, Inc., Rochester, New York)

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Existing information regarding the interrelationship of carotene and tocopherol has been recently reviewed by Quackenbush *et al.* (1). The present experiment adds further data toward the clarification of the mechanism of this interrelationship.

The crystalline carotene used in these experiments was obtained from the S. M. A. Corporation and consisted of approximately 10 per cent α -carotene and 90 per cent β -carotene. The vitamin E was a molecular distillate from soy bean and cottonseed oils; the diets, Nos. 10 and 18, were modifications of the U.S.P. diet for assay of vitamin A depletion. These conditions and the general procedure were as described in a previous article (2).

EXPERIMENTAL

Exploratory assays with carotene gave results which were inconsistent with those for vitamin A. Thus, 4.2 γ of carotene daily, with and without the daily addition of 5.0 mg. of tocopherols, afforded in 28 days nearly equal average growths, 65 and 66 gm. The growth induced by 1.1 γ of carotene alone was *depressed* from 41.8 gm. in 28 days to 27.7 gm. in the group receiving 5 mg. of tocopherols daily. The high response of the group receiving no added tocopherol was due in this early work to the use of Diet 10 (U.S.P. diet containing Wesson oil). The values, which corresponded to those expected when the assays were extended over a much wider range of dosage, are reported here to emphasize the need for searching for the appropriate conditions if the optimum synergy is to be detected.

The most sensitive carotene intake for young rats weighing about 100 gm. has been 0.8 to 1.0 γ daily in our experiments. With less carotene the effect is greater, but too many animals die during assay. An early experiment with 0.8 γ of carotene and Diet 18 is shown in Table I. Here, losses in weight of 6 to 22 gm., terminating in death for rats receiving 0.05 mg. or less of tocopherol daily, become gains of 25 to 32 gm., with the whole group surviving when 0.5 to 1.5 mg. of tocopherols are included. The experiment has recently been repeated (Table II) with Diet 18, which has

* Communication No. 52 from the Laboratories of Distillation Products, Inc.

been compounded with a tested, high vitamin, brewers' yeast and other B vitamins. Here, 1.2 γ daily of carotene were able to support life in the absence of added vitamin E, but gave 23 gm. more growth when the optimum supplement of 0.5 mg. of tocopherols was included. Tables I and II both record that growth is less powerfully augmented with high doses of tocopherol, 1.5 to 5.0 mg. daily.

TABLE I

Effect of Varying Quantities of Tocopherol on Growth Induced by Carotenes (0.8 γ per Day) in Standard Vitamin A Bioassay

Diet No.*	Tocopherol supplement	No. of rats	Beginning weight	Gain or loss in weight		
				28 days	35 days	Average at 35 days
	mg. per day		gm.	gm.	gm.	gm.
18	0.0	4 F.	97.5	-12.7 (1)†	All dead	
		6 M.	107.8	-22.0 (2)	-16 (3)	
18	0.025	4 F.	93.7	2.5	-5.7 (1)	-16.1
		7 M.	113.8	-23.3 (4)	-26.5 (5)	
18	0.050	4 F.	100.7	10.8	12.0	-6.2
		5 M.	110.0	-21.7 (2)	-24.3 (2)	
10	0.09‡	4 F.	98.0	-17.0 (1)	-33.0 (2)	-35.5
		7 M.	104.3	-13.5 (5)	-38.0 (5)	
18	0.15	4 F.	90.5	10.3 (1)	13.0 (2)	11.8
		4 M.	100.7	17.0 (2)	10.5 (2)	
18	0.50	4 F.	102.2	25.3 (1)	25.3 (1)	28.7
		5 M.	110.0	29.3	32.0	
18	1.5	4 F.	97.3	22.0	25.7	23.5
		4 M.	103.2	22.7	21.3	
18	5.0	4 F.	103.7	12.3	8.8	9.6
		4 M.	105.5	7.3	10.3	

* Diet 18 is the u.s.p. vitamin A-deficient diet containing olive oil (vitamin E-free) as the vegetable oil specified. Diet 10 is the same except that Wesson oil is employed.

† The figures in parentheses represent the number of rats that died during the experiment.

‡ It was calculated that this group would receive approximately 0.09 mg. of tocopherol per rat per day from the cottonseed oil of the basal diet.

The effect of vitamin E with larger doses of carotene is shown in Table III. The weight improves only 15 gm. during the feeding period with the optimum tocopherol addition, but there is a marked improvement in maintenance of growth during the 20 days after the carotene supplement has been stopped, with survival time increasing from 53.5 to 79 days.

To dispose of the possibility that the synergy could be merely the arithmetic sum of the separate growth-promoting powers of the two vitamins, the effect of tocopherols on very high levels of carotene was explored with

both depleted and normal undepleted rats. The assay on the depleted rats was uneventful, all the animals surviving. When 30 γ of carotene

TABLE II

*Effect of Tocopherols and Ascorbic Acid on Growth Responses Induced by Carotene (1.2 γ per Day)**

Group No.	Tocopherol supplement	No. of male rats	Original weight	Average gain or loss in weight			
				12 days	20 days	28 days	36 days
	mg. per day		gm.	gm.	gm.	gm.	gm.
I	0	12	107.3	6.9 (3)†	16.4 (3)	23.5 (5)	27.2 (5)
III	0.025	10	114.7	15.1 (1)	23.3 (1)	30.4 (3)	37.0 (3)
IV	0.05	10	109.4	12.8 (2)	22.4 (2)	37.0 (2)	44.1 (3)
V	0.15	11	103.0	14.7 (2)	27.0 (2)	37.0 (2)	38.0 (2)
VI	0.5	11	115.4	17.6 (3)	30.9 (3)	43.9 (3)	50.6 (3)
VII	1.5	11	111.3	9.3 (4)	24.7 (3)	37.1 (3)	43.9 (3)
VIII	5.0	11	101.8	9.9 (1)	10.8 (1)	15.6 (2)	21.9 (3)
I-A	0	6	116.5	-5.5 (2)	9.0 (3)	15.0 (5)	18.5 $\pm$ 3.6 (5)
I-B	0 + 1 mg. ascorbic acid per day	8	115.5	3.8 (2)	12.0 (3)	25.1 (3)	29.0 $\pm$ 4.6 (3)

* Based upon the assumption that the *E* (1 per cent, 1 cm.) (450 $m\mu$) equals 2200 for the carotenes present in this solution.

† The figures in parentheses represent the number of rats that died during the experiment.

TABLE III

Growth and Survival Time Induced by 4.7 γ per Day of Carotene and Influenced by Tocopherols (Diet 18)

Group No.	Tocopherol supplement	No. of male rats	Growth response*		Survival time	
			28 days	48 days	Average	Standard error
	mg. per day		gm.	gm.	days	days
VIII	0.0	9	51.6 $\pm$ 4.6	45.9 $\pm$ 7.9	53.5	4.6
IX	0.05	7	63.7 $\pm$ 5.9	60.8 $\pm$ 7.2	59.1	2.2
X	0.15	7	66.3 $\pm$ 6.1	68.4 $\pm$ 7.8	76.5	4.4
XI	0.5	7	66.9 $\pm$ 2.9	64.4 $\pm$ 6.0	78.0	3.1
XII	1.5	8	52.9 $\pm$ 7.3	69.8 $\pm$ 8.6	79.0	2.6

* The carotene supplement was fed daily from depletion for 28 days, while the tocopherol supplement was continued daily until death occurred.

were fed daily, on a daily dosage of tocopherol of 0.0 mg. the gain in weight in 28 days was 125 gm.; on 0.5 mg., 129.8 gm.; on 1.5 mg., 129.1 gm.; on 5.0 mg., 134.1 gm.

For the other high carotene experiment, normal weanling rats were transferred to Diets 10 and 18 and fed 0.3 γ of carotene daily and in sub-groups, varying quantities of tocopherols for 5 weeks. All the groups were then given 30 γ of carotene daily for a further 5 weeks; that is, until termination of the experiment. The growth responses are listed in Table IV.

TABLE IV
*Growth of Weanling Male Rats on Vitamin A-Free and Vitamin E-Free Diets
Supplemented Daily with 0.3 γ of Carotene for First 5 Weeks
and with 30 γ for Next 5 Weeks*

Diet No.	Tocopherol supplement <i>mg. per day</i>	No. of rats	Gain in weight					
			0.3 γ carotene			30 γ carotene		
			1 wk.	3 wks.	5 wks.	1 wk.	3 wks.	5 wks.
			<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
18	0.0	9	27.0	36.3	29.8	15.2	61.3	100.8
	0.015	9	26.3	34.7	30.2	24.4	67.5	111.4
	0.05	9	24.3	39.5	41.2	27.7	68.4	111.8
	0.15	9	26.9	41.4	47.3	27.0	77.0	119.0
	0.5	9	23.0	46.8	56.4	22.5	68.9	113.3
	1.5	9	27.9	53.6	58.0	27.0	80.6	122.1
	5.0	9	26.0	46.2	48.3	23.8	79.2	128.0
10	0.0	8	22.4	32.2	31.7	20.4	69.2	115.9
	0.15	8	21.2	41.3	43.4	23.2	76.9	122.3
			Without carotene supplement					
18	0.0	7	27.1	29.7	21.2	All dead		
	0.15	7	25.3	26.3	22.5			

DISCUSSION

There is not the space to comment on all the information available from Table IV, but we consider that the trends in this and other tabulations become related and intelligible under the following postulates: (a) Small quantities of tocopherols are synergistic with small and moderate intakes of carotene. Larger quantities of tocopherols are neutral or antagonistic towards small intakes of carotene. (b) There is a definite ratio between carotene and tocopherol intakes; so that the potency of large quantities of carotene benefits from increased quantities of tocopherol. (c) The tocopherols are synergistic with other dietary factors at concentrations higher than the optimum for carotene. They are also useful to the rat without relation to the other vitamins.

Applying these postulates to Tables I and II, one notes the depressing effect of high levels of tocopherol, because in these assays the supply of

carotene is the limiting and only growth factor involved. In Table III a high tocopherol supplement causes only a slight repression of growth because 4.2 γ of carotene are somewhat above the marginal supply and a small depression of synergy does not leave the animal sensibly short of vitamin A. The high carotene levels of Table IV are definitely benefited by excess tocopherols. The fate of part of the carotene is now immaterial and the rat is able to use the extra tocopherol in a primary manner or as a protective agent for other dietary factors. In any event, the experiments indicate that the sparing action of vitamin E for carotene is the main phenomenon. The same rigid proof was not adduced in the previous paper on the vitamin A-vitamin E synergy,¹ but it is believed that the present experiments could be considered to apply to vitamin A also.

No experimental evidence has been advanced here that the sparing action observed in the rat occurs in other animals and humans. Assuming that it does (and much evidence to this effect will be presented elsewhere), we may examine the magnitude of the saving of A vitamins that would be involved by the adoption of vitamin E supplements.

The figures to be considered first are those applying to the rat under the conditions of the experiment. Assuming that Diet 10 contained 0.2 mg. of tocopherols per 10 gm., which is about the daily consumption per rat, and that the level was to be raised artificially to 0.5 mg., then 0.8 γ of carotene would have the effectiveness of 1.0 γ ; 0.3 mg. of tocopherols would "create" 0.2 γ of carotene. However, the diet of humans and most animals does not contain 5 per cent of vegetable oil relatively rich in vitamin E; so that it is worth while to consider the sparing action on poorer diets containing, say, 0.1 mg. of tocopherols per 10 gm. The data have been estimated from the curves of Fig. 1 and listed in Table V.

¹ It is interesting to compare the nearly equivalent growth response at optimum synergy for various forms of vitamin A. Data from this and the previous paper have been tabulated here. The figures happen to show a relative biological potency of

Kind of vitamin	Daily supplement	Tocopherols, optimum daily supplement	Average weight gain in 28 days	Estimated vitamin equivalents for equal gains in weight	
				γ	ratio
Vitamin A OH, crystalline.....	0.57	0.3-0.5	50.5	0.5	1.0
Vitamin A acetate, crystalline.	0.40	0.15	44.1	0.4	0.8
Carotene.....	1.2	0.5	43.9	1.2	2.4

approximately 2:1 for vitamin A and carotene in support of current theories of carotene conversion. Much more experimentation is required before the relative potencies, dietary and intrinsic (that is, under the influence of identical absorption factors, Ψ), of these forms of the A vitamins are known with certainty.

Table V suggests that relatively small quantities of extra carotene are made available by extra vitamin E if this is administered under usual dietary circumstances. However, more optimistic deductions can be made from a plot derived from all the assays of this paper, as in Fig. 2. Here it is seen that the growth of rats on the four dose levels of carotene explored (0.8 to 30 γ daily) without tocopherols falls well enough on a straight line,

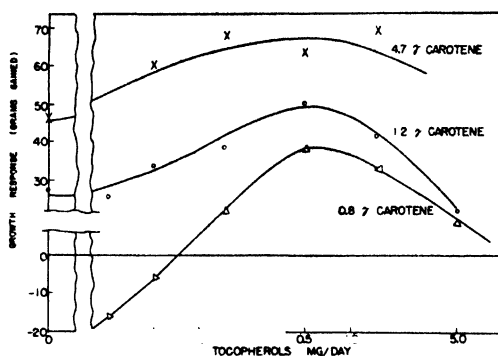


FIG. 1. Influence of various levels of tocopherol intake on the growth response induced in vitamin A-depleted rats by carotene.

TABLE V

Actual Quantity (X) of Carotene Which Will Be Required with (Y) Mg. of Tocopherol to Give Growth Equal to (Z) Micrograms of Carotene with Optimum (0.5 Mg.) Tocopherols

The values are given in micrograms.

Diet contains Y mg. of tocopherols	Z = 0.8		Z = 1.2		Z = 4.7	
	Quantity X required	Carotene "created"*	Quantity X required	Carotene "created"	Quantity X required	Carotene "created"
0.0	2.0	1.2	5.0	3.8	10.0	5.3
0.05	1.5	0.7	2.5	1.3	7.0	2.3
0.10	1.2	0.4	2.0	0.8	5.0	0.3
0.20	1.0	0.2	1.5	0.3	4.7	0.0

* The quantity of carotene "created" = $X - Z$.

Curve 1 connecting weight gain with logarithm of dosage. Curve 2, connecting the extreme assays, indicates the growth to be expected when 0.1 mg. of tocopherols is included in the diet. Curves 3 and 4, drawn through points on the ordinate for 1.2 γ of carotene, show two experimental weight gains which have been obtained from 0.3 mg. of mixed tocopherols (Curve 3) and an even more complex mixture.² Here the covitamin E complexes

² See Paper III of this series.

have "created" 3.2 to 5.4 γ of carotene from an actual 1.2 γ (A and B of Fig. 2). If we consider as conservative figures those in bold face in Table V and as optimistic those just given, it is evident that 1 mg. of tocopherols can "create" 2 to 10 γ of carotene for the rat on a deficient diet. In terms of large commercial quantities, it is seen that to make available 10 trillion (10^{13}) units more of vitamin A from carotene in the American diet, 0.6 to 3.0 million kilos of tocopherols would be required, rather fantastic quantities. Almost identical quantities can be deduced for the tocopherol required to spare or "create" 10 trillion units of vitamin A by admixture with fish liver oils.

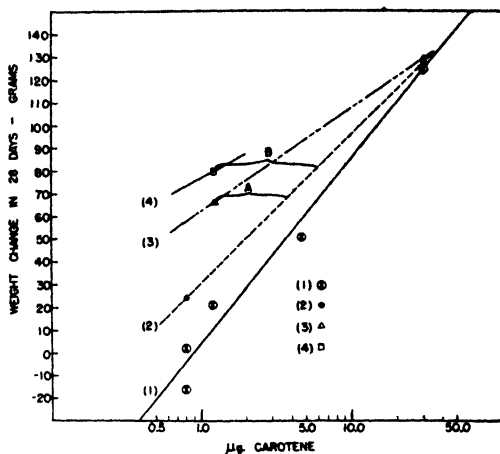


FIG. 2. The dose-response relationships of carotene and various covitamin effects. Curve 1 carotene alone, Curve 2 carotene plus 0.1 mg. of tocopherols per day, Curve 3 carotene plus 0.3 mg. of tocopherols per day, Curve 4 carotene plus 0.25 mg. of tocopherols plus 1.0 mg. of palmityl ascorbic acid per day. A and B represent quantities of carotene apparently "created" by covitamin E complexes.

There are, however, important adjustments to be made in the calculations when one passes from a consideration of rat to human dietaries. It will be suggested in Paper III that the tocopherols spare vitamin A chiefly because they are intestinal antioxidants. It is evidently their absolute concentration in food that is significant and not their relation to body weight or, within limits, to the quantity of carotene present. Humans eat 10 times less in relation to body weight than do rats, but consume an equal or larger relative quantity of carotene. The amount of tocopherols required to create a unit of carotene would thus be diminished 10 to 100 times. The revised calculation assumes a human food intake of 700 gm. of dry weight daily and an optimum tocopherol intake of 0.5 mg. per 10 gm. (the ratio found for the rat), from which the total daily optimum intake

of vitamins E is seen to be about 35 mg. per person. If it is assumed also that the poorest diet contains 0.1 mg. per 10 gm., then the optimum human supplement would be 28 mg. of tocopherols daily. The vitamin A levels in various income brackets have been estimated to extend from 1500 U.S.P. units daily to 3500 units (3). The tocopherol supplement to vitamin A ratio would thus be about 28 mg. to 2500 units. Actually, the exact figure is unimportant, for if the vitamin A level were dangerously low, the tocopherol would impart maximum benefit; and if it were high, the vitamin E would "create" most vitamin A. To achieve this balanced protection, the per capita requirement of tocopherols would be about 10 gm. annually, by no means a fantastic figure.

These very tentative calculations would not be presented were it not for their possible significance in the present food situation and for the fact that they can probably be checked by direct clinical experiment. Indeed, the calculations should promote the experiment. For instance, the increase of vitamin A in blood plasma and tissue (biopsy) following medication with vitamin A, with and without vitamin E, and the spontaneous increase in vitamin A with vitamin E alone can readily be determined and correlated with the rat data. There is little hope of doubling the world's external resources of vitamin A in the next year or so; clinical measurements should show whether or not it is feasible to double or treble those resources internally by the use of extra covitamin E.

SUMMARY

The vitamin A activity of carotene is markedly influenced by the tocopherol intake of the experimental rats. Approximately 0.5 mg. of natural mixed tocopherols is the optimum daily dose to demonstrate the sparing action of vitamin E on carotene.

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COVITAMIN STUDIES

III. THE SPARING EQUIVALENCE OF THE TOCOPHEROLS AND MODE OF ACTION*

By K. C. D. HICKMAN, MARIAN WOODSIDE KALEY, AND PHILIP L. HARRIS

(From the Laboratories of Distillation Products, Inc., Rochester, New York)

(Received for publication, October 4, 1943)

Following the verification of a general synergy between the tocopherols and the A vitamins (1, 2), we have sought to make quantitative comparisons of the sparing powers of pure individual E covitamins and covitamin esters. The substances chosen include the synthetic *dl*- α - and *dl*- γ -tocopherols in free and esterified condition, the natural α -, β -, and γ -tocopherols in various states of combination, ascorbic acid, hydroquinone, and some of their derivatives.

EXPERIMENTAL

The sparing action of these substances has been studied by the method recently described. In the present instance, the synergies were compared at the expected optimum concentration of covitamin, about 0.15 to 0.3 mg. per day per rat, and not over the extended series of concentrations required in the exploratory work. Thus, in a single experiment in which, for example, ten substances are compared, a group of 100 or more rats would be depleted on Diet 18 (the U.S.P. vitamin A-free diet compounded with vitamin E-free olive oil), all fed a uniform daily supplement of crystalline vitamin A (0.5 to 0.7 γ) or carotene (0.8 to 1.2 γ), and then divided into groups of eight to twelve animals, each subgroup receiving the same molecular equivalent of a different covitamin. Two control groups, one receiving neither vitamin nor covitamin, the other no covitamin, would usually be included. We have been impressed with the uniformity of response and the ease with which the values could be duplicated and, thus, checked. It was as though an unrecognized factor contributing to the erratic behavior of vitamin A assays, namely the quantity of the covitamin occurring in the diet, was coming under control. The experimental results are recorded in Table I, where it will be seen that: (1) the sparing action is a property of the free tocopherols and only to a smaller extent of their esters; (2) the separate natural α -, β -, and γ -tocopherols are equally active within the error of experiment; (3) the mixture, as distilled from edible oils, is slightly more active than any of the tocopherols separately. The effect is discussed fur-

* Communication No. 53 from the Laboratories of Distillation Products, Inc.

ther below; (4) two sparing agents, palmityl ascorbic acid and the tocopherols, acting in unison exert a greater synergy than any of the agents separately.

TABLE I
Effect of Various Covitamins on Utilization of Vitamin A and Carotene

Covitamin	Crystalline vitamin A						Crystalline β -carotene (S. M. A.)			
	Daily dose		No of rats	Average weight gains in			Daily dose		No of rats	Average weight gain in 36 days
	Covitamin	Vitamin		16 days	28 days	36 days	Covitamin	Vitamin		
	mg.	γ		gm	gm	gm.	mg	γ		gm.
Control group	0.0	0.57	10	18.1	27.8	30.1	0.0	0.79	10	1.9
Distillate, natural tocopherols*	0.13†	0.57	12	35.1	52.9	57.7	0.42†	0.79	10	30.8
Natural α -tocopherol, pure	0.15	0.57	10	24.3	38.9	48.9	0.50	0.79	10	26.8
Natural β -tocopherol, pure	0.15	0.57	10	31.4	40.9	48.8	0.50	0.79	10	30.4
Natural γ -tocopherol, pure	0.15	0.57	10	32.0	41.5	45.8	0.50	0.79	10	30.3
Natural α -tocopheryl acid succinate	0.15	0.57	10	27.2	32.2	35.0				
Natural α -tocopheryl-quinone	0.15	0.57	10			47.9				
Natural α -tocopheryl-hydroquinone triacetate	0.15	0.57	10			41.0				
Hydroquinone	0.15	0.57	10			30.8	0.13	0.79	10	3.3
Lauryl hydroquinone.	0.15	0.57	10			54.5	0.35	0.79	10	27.9
p-Aminobenzoic acid	0.10	0.57	10			30.8				
Ascorbic acid							0.10	0.79	10	7.4
“ “							1.0	0.79	10	10.9
“ “							10.0	0.79	10	20.3
Control group							0.0	1.2	10	21.6
Distillate, natural tocopherols.							0.25†	1.2	10	63.1
dl-Tocopheryl acetate, synthetic							0.30	1.2	10	24.3
Palmityl ascorbic acid..							1.0	1.2	10	50.5
“ “ “ +										
distillate, natural tocopherols							1.0	1.2	10	80.8
							0.25†			

* From molecular distillation of mixed cottonseed and soy bean oils.

† These quantities were originally believed to have been 15 per cent greater; namely, 0.15, 0.5, and 0.3 mg by the method of physical analysis then in use.

DISCUSSION

Davies and Moore (3) in their description of vitamin A-E synergy in the liver concluded that the increased storage was due to repression of oxidation by the tocopherol which behaved as an antioxidant. "The tocopherols are higher substituted members of the class of hydroxy-aromatic substances of which hydroquinone is a lower and less hydroxylated member . . . often used for stabilizing solutions of vitamin A in fats. The tocopherols may possibly have a similar action under physiological conditions." Evidence that the synergy is due to repression of oxidation is furnished by the effect of ascorbic acid, lauryl hydroquinone, and other substances of established antioxidant character, in sparing the A vitamins. Again, the action of the tocopherols is repressed by esterification. It is well known from *in vitro* experiments that the protective action of aromatic antioxidants is destroyed when the reactive group ($-\text{OH}$) is covered. The residual activity *in vivo* may be attributed to hydrolysis. There is good reason to believe that the effect is exerted chiefly in the intestinal tract. Quackenbush and associates (4) have already stated "... in promoting a biological response to carotene the tocopherol functions as an antioxidant in the gastrointestinal tract rather than as a vitamin regulating some phase of metabolism in the tissues." Our own evidence for intestinal action is the alteration of synergy when the vitamin and covitamin are given at different times or by different routes. If the effect were physiological in nature as through the stimulation of a nerve or endocrine mechanism, separation of time or route should be relatively unimportant. If the effect is chemical and confined to a definite locality, simultaneous arrival of the vitamins at this locality will be beneficial.

The data assembled in Table II support the second alternative. Here it is seen that giving the free vitamins on alternate days diminished the synergy to below that of the esterified covitamins. Administering the vitamin E by intramuscular route is inefficient no matter how the vitamin A is introduced. Surprisingly enough, giving the covitamin by the oral route effects a maximum synergy with the vitamin A given parenterally. This accidentally crucial experiment demands an extension of the simple theory based on intestinal antioxis. Evidently, vitamin E or its equivalent, diffusing inwards through the intestinal mucosa, protects the A vitamins even when these are not present in the intestine. It may be assumed as a working hypothesis that that portion of the body reserves of A vitamins which is at any moment circulating in the blood stream (and this portion in turn is in dynamic equilibrium with the body reserves so that wasting one exhausts the other) will be in danger of destruction each time it passes through the vascular system connected with the intestinal wall. The enzymes and oxidizing agents which ordinarily endanger the vitamin

A during digestive absorption will again be dangerous when the vitamin approaches the same wall from the other side.

There are three ways, similar in effect, but different in mechanism, by which the substances of Table I can spare vitamin A. The true antioxidants inhibit oxidation without necessarily being concerned with the oxygen reserves of the medium. In this class would be placed the tocopherols and lauryl hydroquinone. The second group includes active reducing agents which diminish the oxygen contents or potential of the medium. Their effectiveness becomes greater at high concentration and they themselves are consumed in exerting their function. This class is

TABLE II

Effect of Different Methods of Administration on Covitamin Activity of Tocopherols

O. represents oral; I., intraperitoneal; S., simultaneous; A., alternate days.

Vitamin			Covitamin				Weight gain or loss in 36 days, Diet 18
Supplement	Dose	Route	Supplement	Dose	Route	Time	
	γ			mg.			gm.
Vitamin A acetate.....	0.40	O.	Mixed tocopherols	0.15	O.	S.	51.9
" " ".....	0.40	"	" "	0.15	"	A.	25.1
" " OH.....	0.57	"	" "	0.30	"	S.	55.2
" " ".....	0.57	"	" "	0.30	"	A.	45.7
" " ".....	0.57	"	Control, plain olive oil		I.	*	26.3
" " ".....	0.57	"	Mixed tocopherols	0.15	"	*	35.9
" " ".....	0.57	I.	" "	0.15	O.		53.6
β-Carotene.....	0.79	O.	Control, olive oil		I.	*	2.0
"	0.79	"	Mixed tocopherols	0.50	O.	S.	30.8
"	0.79	"	" "	0.50	I.		12.7
"	0.79	I.	" "	0.50	O.		25.3
"	0.79	"	Control, olive oil		I.		-0.8

* Double the daily dose was given every 2 days.

represented by ascorbic acid in high dosages (1 to 20 mg. per day for the rat). Doubtless, hydrosulfite, zinc dust, or a platinum wire cathode strung through the intestines would achieve equivalent results if such agents were permissible on other grounds. The third class may be divided into two somewhat similar groups. Both comprise two or more synergistic substances acting simultaneously. The simpler is the case in which the antioxidant and the reducing agent are both present, the latter making easier the task of the former. The more complex and perhaps more general case occurs when the primary inhibitory power of the covitamin is itself enhanced by an activator as when tocopherol is assisted by cephalin, phenols,

traces of ascorbic acid, etc. Golumbic and Mattill (5), Golumbic (6), and Swift *et al.* (7), as well as those interested in the commercial stabilization of fats, have compiled a whole roster of tocopherol-promoting agents and the conditions under which they work. Taylor and Jakobsen (8)¹ find that, whereas tocopherol alone will prolong the natural induction period of steam-rendered lard about 20 times, tocopherol with a trace of ascorbic acid will extend the period more than 300 times. Lard which would become rancid in a few hours in warm weather will, when suitably processed with less than 0.01 per cent of complex inhibitor, withstand open contact with the atmosphere for weeks. Nature's task of preserving fat and the vitamins at 37° in the animal body charged with oxygen and oxidative enzymes is infinitely more difficult. In recognizing the rôle which dietary tocopherols, aided by phospholipids, ascorbic acid, and substances yet unidentified, play in protecting the A vitamins, one glimpses something of the miracle that has been performed in preserving from spontaneous oxidation these labile substances in the living animal.

Again on reference to Table I, it is seen that, whereas 1.2 γ of carotene alone afford 21.6 gm. of growth, the same quantity with mixed tocopherols (and the mixture is more potent than the constituents) affords 63.1 gm. and the further addition of palmityl ascorbic acid induces 80.8 gm. Evidently, when the best multiple synergy has been found, the quantities of tocopherols deemed optimum will be less than recorded in these papers. The recent experiment of György (9) on the inhibition of the oxidation of butter yellow by members of the B complex is suggestive of a tocopherol synergy. Enough has been said to show in the study of covitamins that there are activators of secondary and perhaps *n*th status. Our nomenclature, already extended from vitamin E to covitamin E, must be further projected to include various "covitamin E complexes," for it is as members of these mixtures that the E vitamins would seem to make their most economical sparing action.

Fecal Analyses—When the feces of rats or humans are extracted with organic solvents, there results a brown residue which has reducing properties and will furnish an *apparent* tocopherol value when tested by the Furter-Meyer (10), the Emmerie and Engel (11), or the Scudi and Buhs (12) technique. If the extract is purified by various procedures, the test figures gradually diminish until the Emmerie and Engel values approximate those from a rat assay done on the original extract by the antisterility tests. Human subjects from this laboratory showed by bioassay an excretion of 1.2 mg. of α -tocopherol or 2 mg. of mixed tocopherols daily on their usual diet. There is, thus, in normal subjects a small daily fecal excretion of tocopherols and a larger excretion of alcohol-soluble reducing substances.

¹ Also personal communication.

When the subjects are given extra tocopherols, the excretion of vitamin E is increased, as would be expected. What is not necessarily expected, but is of prime importance to the present argument, is that the excretion of the other reducing substances increases also superproportionally to the quantities of tocopherols ingested. These additional reducing materials cannot all be degradation products of the tocopherols, but are evidently labile substances spared by the tocopherols in the alimentary tract (Table III).

TABLE III
Fecal Excretion of Tocopherols

Tocopherols given at start of 5 day collection period (single dose)	Apparent tocopherols excreted in 5 days			
	Bioassay, as α -tocopherol; in parentheses as mixed tocopherols	By Emmerie and Engel method		
		Total gross*	Net increase*	Recovery of test dose
mg.	mg.	mg.	mg.	per cent
None	6.0 (8.5)	63	0	
163	Not assayed	288	225	138
None	" "	58	0	
305	55.0 (80.0)	572	514	168

* Expressed as mg. of α -tocopherol.

TABLE IV
Effect of Tocopherols on Fecal Excretion of Carotene

Tocopherol fed	Carotene fed	Apparent carotene recovered		
		Gross	Net	
mg.	mg.	mg.	mg.	per cent
0	0	5.1		
0	20.0	10.7	5.6- 6.2	28 -31
0	0	4.5		
100	20.0	15.0	9.9-11.5	49.5-57.5
0	0	4.7		

The inhibition of oxidation is again demonstrated when extra carotene is ingested; the quantity surviving in the feces is increased by the tocopherols (Table IV). That it is the destruction rather than the absorption that has been prevented may be assumed from the enhanced physiological activity conferred on carotene by tocopherol in the rat experiments above.

The fresh feces were immediately frozen by solid CO₂ and stored at -20° until required for assay. They were then powdered while frozen and an aliquot transferred to an extractor (13) where they were simultaneously

dehydrated and extracted with benzene. The water which separated and was collected in a graduate gave the dry weight of fecal matter by difference. The benzene extract was transferred to ethanol, filtered, and analyzed. The extracts in the carotene experiments were partially purified by transferal to petroleum ether and extraction by 89 per cent methanol before measurement in the photoelectric colorimeter.

The tocopherols in a day's meals of one of the test subjects (K. H.) were examined by the analytical procedures used for the feces with one alteration. Instead of freezing the food in dry ice and powdering, we dehydrated it from the frozen state by self-freezing in a high vacuum chamber to which it was transferred immediately after serving. In this way, oxidation was minimized and the difficulties presented by the large quantities of water in the beverage portion² were avoided. The food listed in Table V furnished the following readings: by the Furter-Meyer method, 167.0 mg. of apparent tocopherols; by the method of Emmerie and Engel, 12.3 mg.

TABLE V
Constituents of Typical Day's Dietary

Breakfast	Lunch	Dinner
1 egg	1 bowl pea soup	Liver and bacon
2 slices white bread	1 cheese sandwich	Boiled potatoes
2 rashers bacon	1 glass milk	Broccoli
Marmalade	1 fruit cup	Lettuce, tomato salad
$\frac{1}{2}$ pint coffee		Jelly roll
		Coffee

of apparent tocopherols; by rat assay, 4.0 mg. of α -tocopherol equivalent, which may be interpreted as 6 to 7 mg. of mixed tocopherols.

SUMMARY

The three tocopherols, although of different potency towards the sterility syndrome in the rat, are equally effective in sparing the A vitamins. Their synergistic power is increased slightly by mutual admixture and considerably by the addition of selected antioxidants. The tocoquinones are as potent as the tocopherols, presumably after reduction *in vivo*. A simple explanation, as yet only accorded partial proof, is that the sparing action is due chiefly to repression of oxidation in and near the gastrointestinal tract. Carotene and other oxidizable substances are recovered from feces in proportionately greater quantities after simultaneous feeding of tocopherols. The magnitude of increase in recovery found in these experiments,

² These were separately prefrozen in the manner used for dehydrating blood plasma.

nearly 2:1, was of the same order as enhancement of carotene utilization by the rat when fed equivalent quantities of extra tocopherols. Evidence is adduced that further studies of intestinal protective agents will result in further sparing of the A vitamins. It is suggested that the tocopherols which actively control the oxidative changes in the intestines may be found useful clinically in conditions of intestinal derangement.

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A METHOD FOR THE DETERMINATION OF THIAMINE IN THE URINE

By FRANK URBAN AND MELVIN L. GOLDMAN*

(From the Departments of Biochemistry and Medicine, Washington University School of Medicine, St. Louis)

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In this paper the determination of vitamin B₁ in urine after its conversion into thiochrome by means of NaOH and potassium ferricyanide is described. The concentration of thiochrome is measured by determining the amount of light absorbed at its strong absorption band, 368 m μ (1). Since it is comparatively easy to isolate the 365 m μ line of the mercury vapor arc, the latter offers an almost ideal means of measuring the quantity of thiochrome by the amount of light absorbed at 365 m μ where the absorption coefficient and therefore the amount of light absorbed is almost as great as it is at the peak of the absorption band at 368 m μ . The measurement of light absorption by means of a photocell and galvanometer is much more reliable and more delicate than the measurement of thiochrome fluorescence used exclusively so far in the estimation of thiochrome. The chief obstacle connected with the quantitative measurement of the fluorescence is the low fluorescent intensity, making exact observation difficult with the quantities of thiamine found in urine. Furthermore, urine contains blue fluorescent substances which are not thiochrome.

No satisfactory way has been found to differentiate between thiochrome derived from vitamin B₁ in urine and other blue fluorescent substances of urine except by destroying the thiamine with sodium sulfite (2).¹

It is obvious that quantitative recoveries of thiamine added to urine do not constitute proof that the thiamine occurring naturally in urine is determined accurately by a given method. Even after precautions are taken to eliminate all substances found in urine which absorb at 365 m μ , there is finally left in addition to vitamin B₁ a small amount of material which interferes with the vitamin B₁ determination after the latter has been converted to thiochrome, because it also absorbs light at 365 m μ .

It has been found that the conversion of crystalline vitamin B₁ into thiochrome can be completely inhibited after previous treatment with

* Fellow in the Department of Medicine.

¹ In preliminary experiments with Mason and Williams' method of determining the blank by sodium sulfite cleavage of vitamin B₁, the values for thiamine check with the values determined by blocking with benzenesulfonyl chloride. Apparently by sulfite splitting, the non-specific blank remains unchanged when absorption is measured, as Mason and Williams found by measuring fluorescence.

benzenesulfonyl chloride. Attempts are now being made to crystallize this derivative. The above reaction makes it possible to differentiate between vitamin B₁ and other non-specific substances by the simple procedure of measuring the absorption difference between a sample treated with benzenesulfonyl chloride and another not so treated. Evidence has been accumulated to show that benzenesulfonyl chloride does not change the light absorption of the non-specific blank itself.

EXPERIMENTAL

Apparatus—The apparatus (Fig. 1) was set up in a dark room. The source of light was a General Electric 100 watt, T-10, mercury vapor lamp, *A*, with the base down, operated at 110 volts A.C. from a current supplied by a Thordarson voltage regulator, *B*. The lamp was kept in a light-tight chamber equipped with an exit circle of 3 mm. diameter. A suitable combination of lenses directed the beam of light through the absorption vessel (10 cm. polarimeter tube, *E*) into the photocell (General Electric, P-J-22) *H*. An adjustable diaphragm was placed between filter *C* and lens *D*. Monochromatic light of 365 m μ was secured by means of filters (two Corning No. 986 (*C*) and one Corning No. 586 (*F*)). A potential of 45 volts was placed across the photocell ("B" battery) and deflections were measured by means of a Leeds and Northrup d'Arsonval galvanometer of 1×10^{-10} ampere sensitivity, with a critical damping resistance of 26,000 ohms. The scale was placed 12 feet away from the galvanometer. The optical adjustment of the ultraviolet beam has to be such that the galvanometer deflection obtained when no tube is placed in the light path is equal to or greater than the galvanometer deflection obtained when the absorption tube is filled with pure isobutyl alcohol. In the alignment of the apparatus a piece of fluorescent glass (Corning No. 375) is helpful. Also for the adjustment purposes the polarimeter tube may be filled with a dilute quinine sulfate solution (0.5 per cent) which by fluorescence will exactly delimit the optical path in the absorption tube.

Conversion of Crystalline Vitamin B₁ into Thiochrome—Thiochrome was produced from pure crystalline vitamin B₁ (Merck), m.p. 246.5°, by the method of Barger *et al.* (3); that is, the vitamin B₁ was oxidized in alkaline solution by potassium ferricyanide, followed by isobutyl alcohol extraction.

Thiochrome in alkaline solution was found to undergo changes which are reflected in a time rate of change of the light absorption at 365 m μ . This observation is based on a study of the behavior in 20 per cent NaOH of pure crystalline thiochrome with a melting point of 227.5° prepared according to the method of Barger. Stock solutions prepared by dissolving 6 mg. of crystalline vitamin B₁ hydrochloride in 500 cc. of H₂O underwent decomposition while kept at 4°; 22 per cent was destroyed in 5 days. Further-

more a stock solution prepared by dissolving 7 mg. of crystalline thiochrome (m.p. 227.5°) in 20 cc. of isobutyl alcohol underwent decomposition to the extent of 18 per cent when kept at 4° for 12 days.

It follows from these observations that calibration curves have to be constructed from freshly prepared solution and that the thiochrome formed

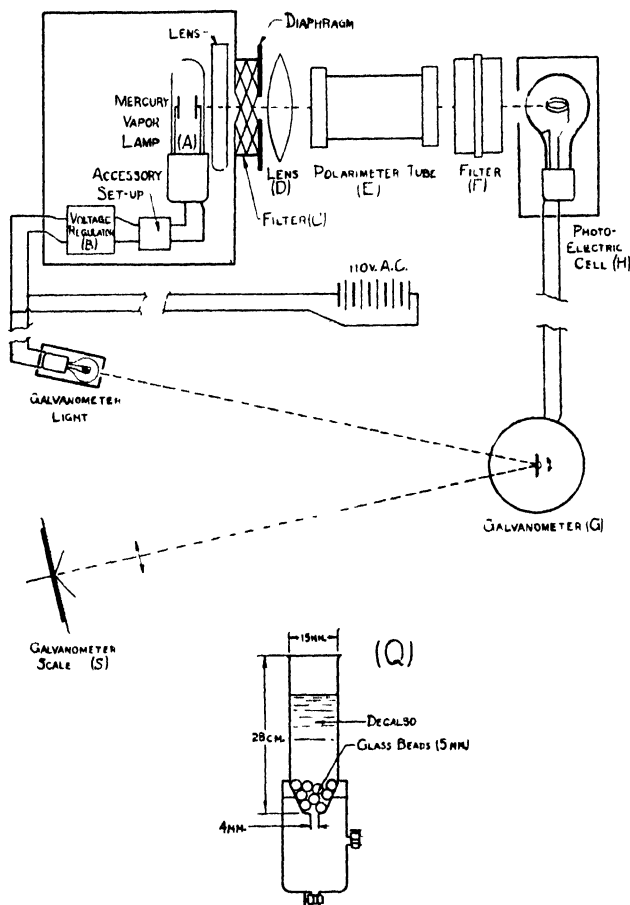


FIG. 1. Apparatus

by the oxidation of vitamin B₁ in alkaline solution must be extracted at a definite time. A calibration curve was constructed by the following technique: 5 cc. samples of aqueous solutions containing 11.13, 21.94, and 33.59 γ of crystalline vitamin B₁ and a control of 5 cc. of water were used.²

² A stock solution containing 12 γ of vitamin B₁ per cc. was weighed, 0.9275, 1.8280, and 2.7990 gm. quantities being used.

To each were added 3 cc. of 20 per cent NaOH, followed by 0.05 cc. of a freshly prepared 1 per cent potassium ferricyanide solution. After exactly 1 minute (stop-watch) 12 cc. of isobutyl alcohol which had previously been measured were added to the aqueous solutions and shaken for exactly 2 minutes. Then the mixture was transferred to centrifuge tubes and centrifuged for $\frac{1}{2}$ minute at a rate of 3000 R.P.M. The supernatant isobutyl alcohol extract was pipetted off. In order to dry the isobutyl alcohol, 3 to 4 gm. of anhydrous sodium sulfate³ were added and left for a period of

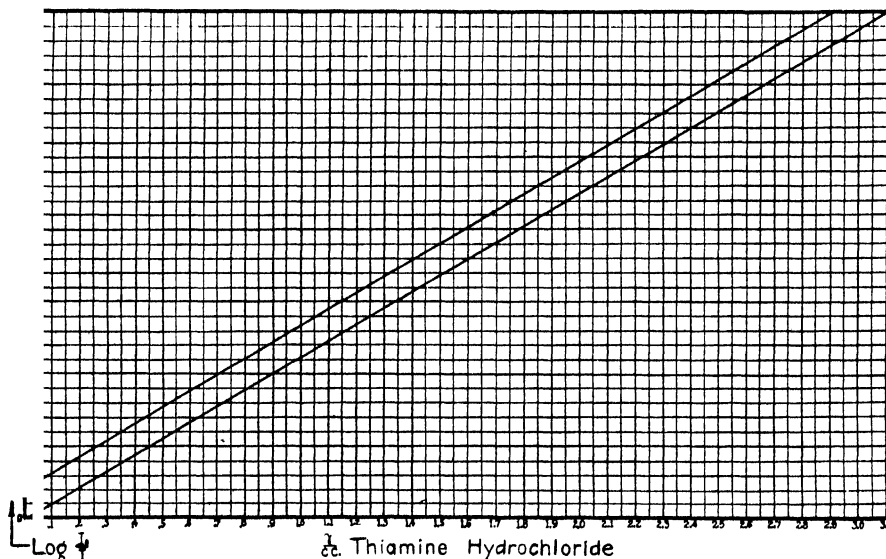


FIG. 2. Calibration curve. The line passing through the origin is the corrected calibration curve. It is obtained by deducting potassium ferricyanide blanks from the actual readings (*i.e.* upper line).

5 minutes. The pipettes used were 10 inches long, so that the isobutyl alcohol did not come into contact with the rubber suction caps.⁴ The

³ Anhydrous Na_2SO_4 will only dry below 31° because of its transition point.

⁴ In this technique there are several precautions that are important: (a) Glass-stoppered flasks and graduates must be used. At no time must any of the solutions of isobutyl alcohol extracts come in contact with rubber, because there are substances in the rubber which have a blue fluorescence and which absorb at 365 m μ . (b) Absolute cleanliness and dryness of the glass disks of the polarimeter tube must be maintained and the tube must be placed in the same position each time a reading is made. (c) An average of ten galvanometer readings should be made at 10 second intervals to correct for fluctuations in voltage not corrected by the Thordarson transformer; *e.g.*, 7.91, 7.90, 7.98, 7.92, 7.88, 7.98, 7.90, 7.92, 7.92, 7.90, average of ten readings 7.92. (d) Before and after each reading the galvanometer should read zero when the light

solution was crystal-clear and when decanted was placed in the absorption tube for determination.

It will be seen that the points in the calibration curve lie on a straight line passing through the origin when the potassium ferricyanide blanks are deducted from the readings (see Fig. 2 and the following values). At a vitamin B₁ concentration (measured in micrograms per cc.) of 0.00 $\log I_0/I$ was 0.038; at 0.93, 0.249; at 1.83, 0.455; at 2.80, 0.673. The specific absorption coefficient as calculated according to the equation $\log I_0/I = K \cdot c \cdot d$ is $68.6 \times 10^3 \text{ cm}^2 \text{ gm}^{-1}$.

Recoveries of Crystalline Vitamin B₁ from Adsorption Column—The determination of vitamin B₁ in the urine is dependent upon its elution from a Decalso adsorption column. Since Hennessy and Cerecedo (4) have found that Decalso will adsorb vitamin B₁ quantitatively, Decalso was used for the adsorption of vitamin B₁ from solutions. The Decalso was activated by washing 50 times with boiling distilled water according to the procedure of Urban, White, and Strassner (5) who found it useful in the activation of Pyrex glass surfaces. Activated Decalso will adsorb vitamin B₁ quantitatively. It should be kept under water until used. 5 to 7 gm. of activated Decalso were used in the column. 2 per cent acetic acid was allowed to flow through the adsorption column by gravity until the filtrate had a pH of 3.5. A solution of crystalline vitamin B₁ acidified by the addition of 2 cc. of glacial acetic acid was allowed to flow through the adsorption column by gravity. A long glass rod was used to eliminate any air bubbles trapped in the column.

By the procedure subsequently adopted in urinalysis, the column was washed with exactly three 10 cc. successive portions of 2 per cent acetic acid. There was a loss of about 10 per cent of the total adsorbed vitamin

path is completely blacked out from the phototube. (e) The determination of I_0 with pure isobutyl alcohol must be carried out with each series of experiments. The following is a sample calculation. Without the tube in place, the galvanometer deflection in cm. was 16.25 for Solution I and 16.70 for isobutyl alcohol; with the tube in place, it was 7.25 for Solution I and 10.01 for isobutyl alcohol.

$$\log \frac{I_0}{I} = \log \frac{10.01 \times \frac{16.25}{16.70}}{7.25} = 0.12822$$

(f) Absolute drying of the isobutyl alcohol extract by anhydrous sodium sulfate must be carried out to prevent destruction of the thiochrome because of the alkalinity of an aqueous extract. (g) The Decalso in the column should be changed after five determinations. (h) A fresh potassium ferricyanide solution should be prepared each day. (i) Before the column is ready to be used again after a determination, a simple test for chloride (dilute nitric acid and 5 per cent silver nitrate) is carried out to make sure the column is free of potassium chloride.

B₁, as shown by analysis of the washings. The vitamin B₁ was eluted with three successive 10 cc. portions of 25 per cent KCl. The KCl eluate after conversion into thiochrome yielded about 90 per cent (see Table I).

Effect of Benzenesulfonyl Chloride on Conversion of Vitamin B₁ to Thiochrome—Benzenesulfonyl chloride was found completely to inhibit the thiochrome formation from vitamin B₁. The experiments which demonstrated this fact were carried out in the following way. To 12 γ of crystalline vitamin B₁ in 5 cc. of water were added 3 cc. of 20 per cent NaOH and 1 drop of benzenesulfonyl chloride. After exactly 3 minutes of shaking in small glass-stoppered flasks, 0.05 cc. of 1 per cent K₃Fe(CN)₆ was added. Exactly 1 minute later 12 cc. of isobutyl alcohol previously measured out were added and shaken for exactly 2 minutes. The usual procedure of centrifugation, pipetting, and drying was carried out. The vitamin B₁

TABLE I

Recoveries of Vitamin B₁ Added to 30 Cc. of Water and Put Through Decalso Column

Vitamin B ₁ added	Recovery of vitamin B ₁	Recovery	Vitamin B ₁ in acetic acid washing	Total vitamin B ₁ recovered
γ	γ	per cent	γ	γ
12	9.79	82.0		
12	10.20	85.0		
24	23.20	96.5		
24	23.70	98.5		
24	21.70	90.0	2.88	24.6
24	19.70	82.0	5.95	25.6
5	4.50	90.0		
3.6	3.96	102.0		
0.96	0.82	85.0		
Average.....		90.0		

recovered when 1 drop of benzenesulfonyl chloride was used was 0 γ ; in the control, 1 drop of water, the recovery was 12 γ .

Isolation of Vitamin B₁ from Urine—According to the procedure of Egaña and Meiklejohn (6), 30 cc. of the 24 hour sample of urine collected with 10 cc. of glacial acetic acid were added to 30 cc. of isobutyl alcohol and shaken for 1 minute and then centrifuged. The isobutyl layer was discarded. The isobutyl-extracted urine was filtered through paper previously washed with hot distilled water. This rendered the urine perfectly clear. To 20 cc. of filtered urine were added 2 cc. of glacial acetic acid and 8 cc. of distilled water. The urine was passed through the column containing activated Decalso and washed with three successive portions of 10 cc. each of 2 per cent acetic acid. The adsorbed vitamin B₁ was eluted by three successive portions of 10 cc. each of 25 per cent KCl.

The KCl eluate was divided into two 10 cc. aliquots. To one 10 cc. aliquot were added 3 cc. of 20 per cent NaOH. After 3 minutes of shaking, there were added 2 cc. of 2 per cent, freshly prepared potassium ferricyanide solution.⁵ After exactly 1 minute, 12 cc. of previously measured isobutyl alcohol were added, shaken for exactly 2 minutes, centrifuged, and the isobutyl alcohol extract (No. 1) dried. To the second 10 cc. aliquot were added 3 cc. of 20 per cent NaOH followed immediately by the addition of 1 drop of benzenesulfonyl chloride. After 3 minutes of shaking, 2 cc. of 2 per cent freshly prepared potassium ferricyanide solution were added. After exactly 1 minute 12 cc. of isobutyl alcohol were added, shaken exactly 2 minutes, centrifuged, and the isobutyl alcohol extract (No. 2) dried.

Isobutyl alcohol contained substances which absorb light of wavelength 365 $m\mu$ in addition to thiochrome. The extinction of isobutyl alcohol Extract 2 deducted from that of Extract 1 equaled the true thiochrome concentration.

Effect of Benzenesulfonyl Chloride on Non-Thiochrome Blank—The simple computation just outlined is only valid if it can be shown that the light absorption at 365 $m\mu$ of the non-specific substances is not changed by treatment with benzenesulfonyl chloride. That these substances are not affected by benzenesulfonyl chloride is shown by the following experiments. In one experiment in which vitamin B₁ was converted to thiochrome, not followed by C₆H₅SO₂Cl treatment, values for log I_0/I were 0.43267 and 0.14459; in another, in which vitamin B₁ was converted to thiochrome, followed by C₆H₅SO₂Cl treatment, the corresponding values were 0.42176 and 0.13964.

Since thiochrome does not react with C₆H₅SO₂Cl, any difference between the experiments with and without treatment by it must be due to an effect of C₆H₅SO₂Cl on the non-specific blank. No effect is seen. The differences are within the experimental error of the method.⁶

Recoveries of Vitamin B₁ Added to Urine—In order to study the efficiency of the method in the recovery of crystalline vitamin B₁ added to urine a number of recovery determinations were made. The five experiments in Table II are examples of many which were carried out.

The recovery was 89 ± 5 per cent. If correction is made for the 10 per cent lost in the acetic acid washing, this would give an average estimate of 99 ± 5 per cent with quantities of the order of 0.15 to 5 γ of vitamin B₁ per cc. of urine.

⁵ The amount of K₃Fe(CN)₆ added should be such that the 10 cc. aliquot is faintly yellow. This usually requires 2 cc. of a 2 per cent freshly prepared solution. An equal amount is added to the benzenesulfonyl chloride aliquot.

⁶ Should any difference be recorded in this control, it can be applied as a correction.

Results

Normal Urine Determination—Table III consists of the vitamin B₁ determinations on normal individuals considered to be on an adequate diet. These consist of laboratory workers and technicians. The samples were voided in the morning at about 10.00 a.m.

TABLE II
Recovery of Vitamin B₁ Added to Urine

Urine used	Vitamin B ₁ added	Vitamin B ₁ recovered	Recovery
cc.	γ	γ	per cent
5	24	20.7	86.0
5	24	20.4	85.0
5	12	10.1	84.5
17.6	10	9.24	92.4
20.0	3	2.84	94.6
Average			88.5

TABLE III
Vitamin B₁ in Normal Human Urine

Subject	Urine used	Vitamin B ₁ per cc. urine
	cc.	γ
J.....	15.0	0.081
M.....	30.5	0.290
"	31.0	0.250
U.....	20.0	0.205
M.....	20.0	0.223
E.....	20.0	0.222
U.....	20.0	0.208
E.....	20.0	0.182
M.....	19.8	0.238
"	20.0	0.261 (24 hr. sample)

SUMMARY

A method for the determination of thiamine in the urine is proposed, based on the use of two principles:

1. The specific light absorption of thiochrome can be measured at 365 mμ by means of a photoelectric cell and galvanometer.

2. The unknown substances occurring in the urine which produce color at 365 mμ are determined by blocking the conversion of thiamine into thiochrome by treatment with benzenesulfonyl chloride. The difference

between light absorption produced without benzenesulfonyl chloride and that obtained with it is a measure of true thiamine in urine.

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EFFECT OF CYANIDE, FLUORIDE, AND MAGNESIUM ON SERUM PHOSPHATASE ACTIVITY DURING HEPATIC DAMAGE

By VICTOR A. DRILL,* J. H. ANNEGERS, AND A. C. IVY

(From the Department of Physiology, Northwestern University Medical School, Chicago)

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Alkaline phosphatase, normally present in the serum, is known to be raised above normal in various types of liver damage (1-5). Although the effects of various inhibitors, such as cyanide and fluoride, have been studied on phosphatases extracted from tissue ((6-8) and others), no observations have been made on the effect of such ions on serum phosphatase activity during hepatic damage. In this paper the effect of cyanide, fluoride, and magnesium ions, alone and in various combinations, was studied to ascertain their effect on the serum phosphatase activity of normal dogs and dogs with hepatic damage. It was found that the increase in serum phosphatase obtained during liver damage was due to a phosphatase that was normally present in the blood in only small amounts.

Methods

Inorganic serum phosphate and alkaline serum phosphatase were determined according to the method of Bodansky, β -glycerophosphate being used as substrate (9). Substrate was also prepared with the following concentration of salts: MgSO_4 0.01 M, NaF 0.01 M, NaCN 0.0001 to 0.1 M. Hepatic damage with a consequent rise in serum phosphatase activity was produced by the following procedures: administration of CCl_4 (10, 11), cholecystectomy (12), production of a bile fistula (13), and in bile fistula dogs in which the biliary tree had become obstructed. The dogs were fed a stock diet consisting of bread, meat scraps, bones, cod liver oil, and yeast. On this diet the serum phosphatase values of normal dogs varied between 4.0 and 6.5 units per 100 cc. of serum.

Results

NaCN, in concentrations from 0.0001 to 0.1 M, had only a slight effect on the activity of serum phosphatase from normal dogs (Tables I and II). In dogs with hepatic damage produced by various procedures the serum phosphatase was elevated above normal, the rise being most marked in cases of biliary obstruction. In these dogs the addition of NaCN pro-

* National Research Council Fellow, 1942-43. Present address, Department of Pharmacology, Yale University School of Medicine, New Haven.

duced an inhibition of phosphatase activity, and as the molarity of the NaCN was increased the loss of activity became progressively greater (Table I). However, as the concentration of NaCN was increased, the serum phosphatase activity decreased to normal values, *but did not go below normal*. This can be seen with NaCN concentrations of 0.01, 0.05, and 0.1 M, when the serum phosphatase activity has decreased to fairly constant values. This effect of NaCN on elevated serum phosphatase was obtained

TABLE I

Effect of Various Concentrations of NaCN on Serum Phosphatase Activity of Normal Dogs and Dogs with Hepatic Damage

Dog No	Operation	Inorganic PO ₄	Phosphatase units per 100 cc. serum NaCN added to phosphatase substrate								
			None	0.0001 M	0.001 M	0.0025 M	0.005 M	0.0075 M	0.01 M	0.05 M	0.1 M
Normal dogs											
		mg. per 100 cc.									
93		4.90	6.32		5.94		5.38		4.60	4.52	4.57
94		4.10	4.62		4.56		3.85		3.15	3.29	2.86
108		4.02	8.83		7.90		3.88		4.78	4.88	5.41
109		5.21	6.00		6.02		5.62		4.59	5.17	5.47
110		3.28	5.02		4.72		4.33		3.96	4.18	4.32
Dogs with hepatic damage											
81	Obstruction	3.49	86.0	84.5	73.5	50.7	23.6	16.0	6.39	4.63	4.53
81	“	3.92	106.2	106.2	95.1	49.6	18.3	9.28	4.66	4.59	4.66
G-8	“	3.20	40.9	41.6	40.8		10.1		6.60	5.05	5.92
G-8	“	4.72	56.7	54.3	47.2	35.8	19.3	15.3	6.30	5.53	5.53
95	CCl ₄ administered	3.40	26.4	24.2		16.8		8.42	5.02	4.86	4.94
97	“ “	4.56	37.2		29.1		11.3	8.84	7.39	5.19	
84	Cholecystectomy	4.59	10.4	9.8		7.3		5.19	4.19	5.23	5.29
102	“	4.26	14.2		10.1		7.8	6.48	5.62	5.06	5.34
103	“	4.05	12.9		9.6		7.3	6.52	4.60	3.86	4.28
105	“	4.38	16.1		10.4		8.0	5.88	5.48	5.94	5.36

in the three types of liver damage studied, and suggests that the increased serum phosphatase during hepatic damage is due to an alkaline phosphatase that is normally present in the blood in only small amounts.

Magnesium sulfate (0.01 M) had only a slight activating effect on serum phosphatase in normal dogs, the average increase being 0.91 unit per 100 cc. of serum (Table II). The addition of NaF also produced a slight increase in phosphatase activity (Table II).

NaCN (0.01 M) inhibited serum phosphatase activity in normal dogs by an average of 1.92 units (29.9 per cent). The NaCN was also effective in the presence of Mg, inhibiting the slight stimulation produced by Mg, and lowering the value to that produced by NaCN alone (Table II). In a few trials with 0.01 M NaF and 0.01 M NaCN, the NaCN again abolished any stimulating effect of the NaF, lowering the value to that obtained with NaCN alone.

These studies were then repeated on dogs with liver damage. Indogs with high serum phosphatase values Mg had only the small stimulating action seen in normal dogs, suggesting that it had no effect on the increased serum phosphatase produced by the liver damage. The effect with NaF

TABLE II

Effect of NaCN, NaF, and Mg on Serum Phosphatase Activity of Normal Dogs and Dogs with Hepatic Damage

Dog No.	Operation	Inorganic PO ₄ mg. per 100 cc.	Phosphatase units per 100 cc. serum Additions to phosphatase substrate					
			None	0.01 M Mg	0.01 M NaCN	0.01 M Mg, 0.01 M NaCN	0.01 M NaF	0.01 M Mg, 0.01 M NaF
93	Normal	4.90	6.32	7.13	4.60	4.75	6.18	6.18
94	"	4.10	4.62	5.51	3.15	3.10	5.07	5.68
108	"	4.02	8.83	9.58	4.78	4.66	10.79	10.12
109	"	5.21	6.00	6.98	4.59	4.78	7.18	7.42
110	"	3.92	6.24	7.38	5.30	5.18	7.24	7.34
95	CCl ₄ administered	6.87	11.3	12.7	6.21	6.54	12.1	13.1
G-8	Obstruction	3.80	30.2	33.8	11.19	11.55	34.0	30.0
81	"	3.92	106.2	106.4	4.66	4.70	102.6	104.8
91	Bile fistula	4.94	22.8	25.6	5.27	5.06	23.8	24.0
84	Cholecystectomy	4.76	8.99	9.47	5.64	5.74	11.2	10.8

and Mg plus NaF was slight, and also similar to that observed in normal dogs. NaCN again inhibited the increased phosphatase activity, even in the presence of Mg.

DISCUSSION

Extracts of non-specific alkaline phosphatase from various tissues, generally characterized as a phosphomonoesterase, are not inhibited by 0.01 M fluoride, while 0.01 M cyanide will produce about 90 per cent inhibition of activity (6-8). Recently Cloetens prepared phosphatase extracts from liver tissue which he fractionated into alkaline phosphatase I and alkaline phosphatase II (8). The activity of alkaline phosphatase I was not in-

hibited by 0.01 M KCN but was reduced by 0.01 M KF. Alkaline phosphatase II, however, was completely inhibited by 0.01 M KCN, and a similar concentration of KF was without effect. Gomori (14) recently prepared an alkaline phosphatase from various tissues which had a specific effect on hexose diphosphate. This hexosediphosphatase was inhibited by fluoride and activated by cyanide.

The alkaline phosphatases of Cloetens and the hexosediphosphatase of Gomori are present in liver. The rise in alkaline serum phosphatase activity observed in dogs with hepatic damage is not due to hexosediphosphatase, as this enzyme will not act on β -glycerophosphate. Neither does the increased serum phosphatase activity correspond to Cloetens' alkaline phosphatase I, for the effects of CN and F are opposite in either case. The effect of CN and F on the increased serum phosphatase activity during hepatic damage does correspond to the effect of these ions on Cloetens' alkaline phosphatase II. However, 0.01 M Mg will activate alkaline phosphatase II 200 to 300 per cent, but has only a very slight effect on serum phosphatase activity of dogs with liver damage (Table II). It does not seem likely that the lack of effect of Mg on serum phosphatase activity is due to the presence of the small amount of Mg in the blood serum. Serum is reported to contain 1 to 3 mg. of Mg per 100 cc. (15), which would correspond to only 0.0008 M MgSO_4 . In the determination of phosphatase activity 1 cc. of serum is added to 10 cc. of substrate, so that the final concentration of Mg is very low.

SUMMARY

1. Sodium cyanide, in concentrations of 0.0001 to 0.1 M, had only a slight inhibitory effect on serum phosphatase activity of normal dogs. When the serum phosphatase values increased as a result of liver damage, the addition of NaCN inhibited phosphatase activity. However, as the concentration of NaCN was increased the serum phosphatase activity decreased to normal values, but did not go below normal. As this result was obtained in three types of liver damage, it suggests that the increased serum phosphatase activity during hepatic damage is due to an alkaline phosphatase normally present in the blood in only small amounts.

2. Magnesium sulfate (0.01 M) and NaF (0.01 M) each had a slight activating effect on serum phosphatase activity of normal dogs. In dogs with high serum phosphatase values Mg and F had only the slight effect noted in normal dogs. A combination of Mg and F did not further increase activity in either case. The addition of NaCN (0.01 M) still produced the characteristic inhibition in the presence of Mg or F.

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ELECTROPHORETIC ANALYSIS OF YEAST EXTRACTS

By KURT G. STERN

(From the Overly Biochemical Research Foundation, New York)

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When pressed yeast is slowly dried in the open at about 30°, the cell membrane is destroyed by autolysis. The maceration of such slowly dried yeast with water at 37° and subsequent filtration or centrifugation yield cell-free extracts which, as a rule, will actively ferment fructose, glucose, and other suitable substrates. Maceration extracts of yeast, prepared in this manner, are often designated as *Lebedev juice*, after their discoverer A. von Lebedev (1). Although the kinetics of fermentation, as catalyzed by such extracts, differ somewhat from those observed with intact yeast cells, especially with regard to the rate of the accumulation and the splitting of hexose phosphate esters, the essential correlations between the various enzymatic components of the zymase complex are preserved to a remarkable extent (2). Studies on the mechanism of alcoholic and glycerol fermentation, performed with such cell-free extracts, have thrown light on many intermediate reactions which cannot readily be observed in work with whole cells but which proceed undoubtedly *in vivo* as *in vitro*. The absence of the cell membrane avoids complications due to permeability factors, the inhibition of fermentation by oxygen (Pasteur reaction) is eliminated, and chemical fractionation leads to purified preparations capable of catalyzing specific intermediate reactions (*cf.* (2)). In recent years, extracts of this type have been employed in research in fermentation almost to the exclusion of the earlier Buchner yeast juice, which requires a hydraulic press for its preparation. Upon dialysis against water, certain coenzymes, *e.g.* cozymase and adenylic acid, and activating metal ions, *e.g.* magnesium and manganese, are removed and the fermentative power of the extracts is lost. It may, however, be restored by the addition of the low molecular activators just mentioned, thus proving that the dialyzed extract still contains, in active form, all the proteins required for the catalysis of fermentation.

The charting of the colloid "spectrum" of unfractionated maceration extracts of yeast, as it reveals itself in electrical and intense gravitational fields under optical observation, is a prerequisite to the isolation of individual components, particularly proteins, by preparative electrophoresis or ultracentrifugation. The present experiments are concerned with the optical study of maceration extracts, prepared from various types of dried yeast preparations, in the Tiselius electrophoresis apparatus. They furnish in-

formation on the minimum number of individual colloidal components in such preparations, on their electrochemical properties, and on their relative concentration. Some of the components thus observed are undoubtedly catalytically inert proteins and perhaps also polysaccharides extracted from the yeast cell. Others, especially the components present in low concentrations, represent possibly intracellular yeast enzymes or the protein moieties of dissociable enzyme systems (*apoenzymes* in von Euler's terminology). Contrary to the belief, widely held, that equilibration of a biological colloid system against suitable buffer solutions across semipermeable membranes will not affect appreciably the "native" electrophoretic pattern, it will be shown that the dialysis of maceration extracts of yeast modifies the original pattern significantly, even if anomalies due to differences in conductivity and hydron concentration between the colloid system and the supernatant buffer solution are taken into consideration.

EXPERIMENTAL

Materials and Preparation of Extracts—Brewers' top and bottom yeasts as well as bakers' yeast were employed for the preparation of the dry yeast samples serving as the source of the maceration extracts. Four batches of dry yeast, derived from two different samples of brewers' bottom yeast, two batches of dry brewers' top yeast, and one batch of dry bakers' yeast were used for extraction. The majority of the experiments was performed on a batch of bottom yeast (Frohberg strain of *Saccharomyces cerevisiae*) kindly furnished by the Hull Brewing Company, New Haven, Connecticut. The yeast was received in the form of a concentrated suspension in beer wort. It was carefully washed by decantation in a cold room, freed from most of the adhering fluid by pressing in a hand press, crumbled, and dried for 2 days in thin layers in the air stream of a Grinnel drier at room temperature. The dry yeast was milled to yield a coarse powder and stored at room temperature. A small batch of Hull's top yeast was prepared in a similar manner. Federal bakers' yeast and Krueger's bottom yeast were obtained already in washed and pressed form. These samples were driven through a coarse sieve, spread in thin layers, and allowed to dry at room temperature in the case of the bakers' yeast and at about 30° in the case of the bottom yeast, three different batches of which were prepared for this work. The age of the dried yeast samples at the time of extraction ranged from 1 month to 2 years.

The maceration extracts were usually prepared essentially according to the original method of von Lebedev (1); *viz.*, by suspending 1 part of dried yeast in 3 parts of tap water at about 45°, macerating the suspension for 2 hours at 37°, and subsequently centrifuging for 30 minutes at about 3000 R.P.M. in the horizontal head of a No. 2 International centrifuge. In some

instances the maceration extracts thus prepared were not sufficiently transparent for study with the schlieren method; they were therefore further clarified by spinning for 10 minutes at 10,000 R.P.M. in the chilled quantity rotor of an air-driven Beams ultracentrifuge of the design of Bauer and Pickels. In one instance (Krueger's bottom yeast, Batch 12) 1 part of dry yeast was suspended in 2 parts of tap water and stirred mechanically for 2 hours at 35–37°, after which 1 further volume of water was added, the stirring continued for another 15 minutes, and the final suspension then centrifuged for 25 minutes at 3300 R.P.M. in the laboratory centrifuge and another 15 minutes at 10,000 R.P.M. in the ultracentrifuge to remove opalescent material. When samples of these maceration extracts were tested for their fermentative power in the Warburg manometric apparatus, not all of them were able to ferment actively glucose or fructose within the short periods employed for observation (1 to 2 hours at 22–23°). One of the samples of dried bottom yeast (Krueger's yeast, Batch 10) which failed to yield an actively fermenting Lebedev juice when macerated in the manner described above, gave a highly active extract when treated with 3 parts of 0.167 M diammonium phosphate solution for 2 hours at 37°, as recommended by Neuberg and Lustig (3). However, even those maceration extracts which were incapable of catalyzing the over-all fermentation of sugars and were therefore deficient with respect to one or several components of the zymase complex contained a number of enzymes in active form; *e.g.*, yellow enzyme, aldolase (according to Dr. Z. Dische), carboxylase, alcohol dehydrogenase, and glycerophosphate dehydrogenase. The color of the native maceration extracts was too deeply yellow-brown and their nitrogen content was too high (8 to 15 mg. per cc.) for direct examination in the Tiselius apparatus. They were therefore diluted 4 to 5 times with the buffer, employed as the supernatant, prior to electrophoresis. For the experiments on dialyzed extracts, freshly prepared Lebedev juice was placed in cellophane tubing and dialyzed for 2 days in the refrigerator against 100 volumes of phosphate buffer of the desired ionic strength and pH. The outside fluid was then used as the supernatant buffer solution in the Tiselius cell. Prior to experiments on undialyzed extracts, the pH and the conductivity of the yeast extract and of the supernatant buffer solution were equalized by addition of acid, base, or suitable dilution. In the experiments with Hull's bottom yeast (Batch 4) the pH was varied from 5.6 to 8.3; sodium phosphate buffers were used and an ionic strength of 0.3 maintained. In the experiments with the other yeast samples, 0.1 M sodium phosphate buffer, containing 188 parts of dibasic and 12 parts of monobasic sodium phosphate (ionic strength, 0.3), was used as the solvent throughout. The pH in these experiments varied only within the limits of 7.70 and 7.97. The pH was determined with a Beckman glass electrode

pH meter and the conductivity was measured at 1° by means of a Shedlovsky conductivity cell and an Industrial Instruments conductivity bridge having a "magic eye" as the indicator instrument. The nitrogen content of the solutions employed in the majority of the experiments amounted to 2.5 mg. per cc. on the average.

Electrophoresis Technique—The Tiselius electrophoresis apparatus employed in this investigation was constructed by the Klett Manufacturing Company, New York, according to the design of Dr. L. G. Longsworth. The electrophoresis cell was equipped with the tall (80 mm.) center section recommended by Tiselius and Longsworth for the optical analysis of colloid systems (*cf.* (4)). It accommodates about 10 cc. of protein solution in a narrow channel of rectangular cross-section (3×25 mm.) which is built up from optically plane glass plates with the aid of acid- and temperature-resistant cement. The sliding ground glass plates of the cell were lubricated with Celloseal, a proprietary preparation containing polymerized castor oil and exhibiting only little change in viscosity over a rather broad temperature range (0–100°).^{*} As light sources, a mercury, high pressure vapor lamp (General Electric, type H-4) and a low voltage, straight single coil tungsten lamp of 1000 lumen intensity were used, depending on the color and degree of transparency of the yeast extracts. The temperature of the water bath surrounding the cell was held constant within narrow limits at 1.3° with the aid of mechanical refrigeration and an electronic relay. The voltage gradients applied to the cell ranged from 1.2 to 4.3 volts per cm. The development of the electrophoretic diagrams was followed visually with the aid of the Philpot-Thovert angular diaphragm and cylinder lens arrangement (*cf.* (4)). When the system had been resolved sufficiently in the electrical field, the diagrams were recorded on panchromatic plates by the schlieren scanning method of Longsworth. The mobilities and relative concentrations of the individual components were computed in the usual manner from measurements on tracings of the original plates enlarged by projection.

Observations and Results

A total of thirty-three electrophoresis experiments was carried out on maceration extracts prepared from eight individual batches of three different types of dried yeast. Twenty-eight of these experiments yielded results suitable for quantitative treatment. The first series of experiments (ten in number) was performed on dialyzed Lebedev extracts prepared in the standard manner from *Hull's dried bottom yeast* (Batch 4). These extracts, after centrifugation in the laboratory centrifuge, were dark yellow and usually clear; their pH was 5.6 to 6.0 and their nitrogen content about 10 mg. per cc. Owing to the appreciable phosphate content of the

^{*} See note on p. 361.

yeast, the conductivity of such extracts was actually somewhat greater than that of the 0.1 M phosphate buffer used as supernatant. Upon dialysis against such buffer solutions, the nitrogen content decreased to about 2 mg. per cc., corresponding to approximately 1.2 per cent protein concentration. The dialysis effected a close equilibration of the yeast extract with the outside buffer solution, both with regard to conductivity and pH. Upon electrolysis of such dialyzed extracts from Hull's bottom yeast in the Tiselius apparatus at pH 5.8 to 8.3, a separation into two groups of colloids occurs within 2 to 3 hours at potential gradients of 2.4 to 4.3 volts per cm. Fig. 1 is the reproduction of a typical electrophoretic diagram recorded in these experiments.

The bulk of the material giving rise to the refractive index gradients observed in the electrophoresis cell (about 80 per cent) remained near the

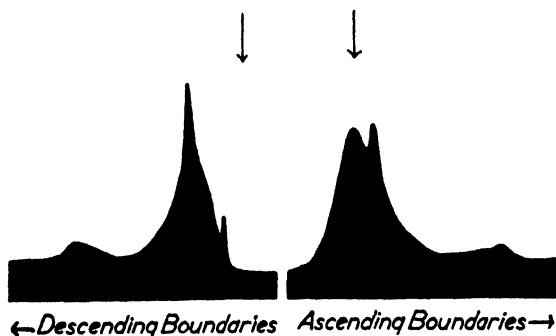


FIG. 1. Electrophoretic diagram of dialyzed maceration extract of dried Hull's bottom yeast, recorded after 205 minutes electrolysis at pH 6.7. Potential gradient, 3.2 volts per cm. The vertical arrows indicate the starting position of the boundaries.

starting position of the initial extract-buffer boundary. In the anode compartment, containing the ascending boundaries, this major peak usually resolved itself into two separate maxima endowed with zero and small anodic mobility respectively. The area under the stationary peak was somewhat smaller than that under the mobile one. It is quite possible that the electroneutral material is a polymer carbohydrate rather than a protein. The second group of colloids, representing presumably protein components of the yeast extract, exhibits an appreciable electrophoretic mobility; in a number of instances this mobile fraction resolved itself into two separate minor components of mobilities of the order of -6 and -8×10^{-5} cm.² sec.⁻¹ volt⁻¹. The degree of resolution of the various maxima in the cathodic limb of the cell, containing the descending boundaries, was appreciably smaller than in the case of the ascending boundaries. In two instances, a minor component with intermediate mobility was observed in

addition to the two main groups just mentioned. Since the chemical nature of these various colloids remains to be determined, they are here designated, for purposes of convenience, in a manner analogous to the practice adopted by Tiselius in his studies on blood sera (5). The fraction with the smallest anodic mobility is called the γ fraction, the one with the highest mobility is labeled the α fraction, while the one with intermediate charge is called the β fraction. Numerical subscripts serve to differentiate between the individual members of these groups, the lower numerals denoting the components with the lower mobility. In some experiments on yeast extracts, a minor, well defined component of small cathodic mobility within the pH range employed was observed, as a rule in the cell compartment occupied by the ascending boundaries. This component, which must have an isoelectric point higher than pH 8, is designated as the δ fraction. In contrast to the stationary δ -boundary, first observed by Tiselius in his work on blood sera and later identified by him and Longworth (*cf.* (4)) as a boundary anomaly due to the interaction of buffer salts with protein components, the present δ -boundary is most likely caused by a real component of the yeast extracts, since it has a small but definite cathodic mobility. Furthermore, this boundary has always the form of a sharply defined maximum in contrast to the rounded off anomalous δ - and ϵ -boundaries observed with blood serum. A final decision on this point will have to await the result of electrophoretic separations.

The data obtained on analysis of the electrophoretic diagrams of dialyzed maceration extracts from Hull's bottom yeast are presented in Table I. It will be noted that there is no consistent trend towards an increase in mobility of the various components upon decrease in the hydron concentration within the pH range employed. The slope of the pH-mobility curve is primarily a function of the chemical nature of a protein. Thus, the slopes of the mobility curves of the protein components of horseserum differ greatly, that of the γ -globulin being much smaller than that of α -globulin or albumin (Tiselius (5)). In view of the relatively small number of experiments performed thus far on yeast extracts at different pH values, all that can be said at this time is that the pH-mobility curves of the colloids present in them are surprisingly flat; their exact slope remains to be ascertained. No statistical significance is attached to the mean concentration and mobility values given at the bottom of Table I; they are merely given as a convenient means of summarizing the results thus far obtained.

A subsequent series of nine experiments was concerned with the electrophoresis of undialyzed Lebedev extracts from the same batch of dried, Hull's bottom yeast. The nitrogen content of the solutions was made similar to that of dialyzed extracts by dilution. As illustrated in the typical diagram reproduced in Fig. 2, the pattern given by the undialyzed

yeast extracts is considerably more complex than in the case of the dialyzed solutions (compare with Fig. 1). In addition to a large γ fraction with zero or low mobility, and the α fraction endowed with a relatively high

TABLE I

Electrophoretic Analyses of Dialyzed Maceration Extracts of Dried Brewers' Bottom Yeast (Hull's, Batch 4)

Ex- peri- ment No.	pH*	Boundaries†	Relative concentrations of individual components, per cent							Electrophoretic mobility, cm. ² sec. ⁻¹ volt ⁻¹ × 10 ⁴					
			δ	γ ₁	γ ₂	γ _{total}	β	α ₁	α ₂	α _{total}	γ ₁	γ ₂	β	α ₁	α ₂
256	5.76	A.				88		6	6	12		-1.14		-6.14	-8.21
		D.				88		4	8	12		-0.67		-5.24	-7.22
257	6.1	A.		27	61	88		10	1	11	+0.35	-0.88		-7.47	-8.27
		D.	3			68				29					
255	6.7	A.		43	48	91		4	5	9	+0.10	-1.03		-6.71	-8.26
		D.	5	24	60	84				11					
258	7.18	A.		24	60	84				15	0	-0.67			-8.47
		D.		20	69	89				15	0	-1.23			-8.31
252	7.6	A.‡		34	34	68	13			6	0	-1.57	-2.58		-8.71
		D.				85				15		-0.80			-7.54
259	8.3	A.		34	44	78	13			9	-0.02	-1.01	-2.63	-6.33	
		D.		17	49	66	19			15	-0.08	-0.85		-6.05	
Mean values		A.		32	49	83				10	0	-1.05		-6.66	-8.38
		D.		(20)	(60)	80				16	0	-0.89		(-5.65)	-7.69

* Solvent, sodium phosphate buffer, $\mu = 0.3$.

† A., ascending; D., descending.

‡ Additional component (13 per cent) of slightly higher mobility than that of the β component present.

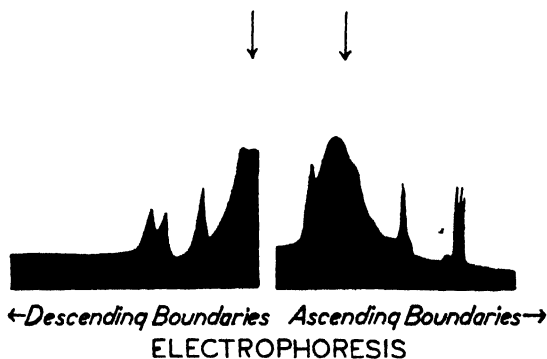


FIG. 2. Electrophoretic diagram of undialyzed, diluted maceration extract of dried, Hull's bottom yeast, recorded after 387 minutes electrolysis at pH 7.86. Potential gradient, 1.8 volts per cm.; nitrogen content, 2.3 mg. per cc.

TABLE II
Electrophoretic Analyses of Undialyzed Maceration Extracts of Dried Brewers' Bottom Yeast (Hull's, Batch 4)

Relative concentrations of individual components, per cent			Electrophoretic mobility, cm. ² sec. ⁻¹ volt. ⁻¹ × 10 ⁶																			
Experiment No.	pH*	Boundary†	Relative concentrations of individual components, per cent										Electrophoretic mobility, cm. ² sec. ⁻¹ volt. ⁻¹ × 10 ⁶									
			δ	γ ₁	γ ₂	ρ ₁	ρ ₂	ρ ₃	α ₁	α ₂	α ₃	α _{total}	δ	γ ₁	γ ₂	ρ ₁	ρ ₂	ρ ₃	α ₁	α ₂	α ₃	
264	5.6	A.																				
260	5.65	"																				
265	6.12	"	11									6	5		+1.53							
266	6.12	"		44	32	76	6	3	1	10	1	12	1	14	+1.13	+0.54	-5.01	-5.31	-6.03	-6.98	-7.41	
267	7.81	D.†	18	20	71	4	6	1		11	15	3	18		-0.03	-0.88	-4.47	-4.79	-5.52	-6.61		
268		A.	8	47	21	68				9	2	1	11	14	-0.37	-2.56	-3.59	-4.85	-5.51	-6.13		
269		D.	52	23	75					11	3	2	8	13	-0.81	-1.97	-3.99	-4.36	-4.83	-5.33	-7.86	
270	7.85	A.	11	38	26	64				11			14		+1.84	0	-3.08	-3.96	-4.55	-5.41	-6.75	
271		D.				66				13	5	4	10	19	-0.66	-0.66	-2.83	-4.36	-5.73	-6.34	-7.01	
272	7.86	A.	8	41	25	66				12	4	4	5	13	+1.57	+0.33	-3.25	-4.93	-6.01	-6.21	-6.48	
273		D.		25	43	68				13	5	3	11	19	+0.23	-0.63	-2.72	-6.01	-6.27	-6.48		
274	7.86	A.	7	44	24	65				13	8	7	15		+0.99	-0.02	-4.06	-4.66	-4.92	-5.47		
275		D.		37	33	70				12	8	10	18		+0.27	-1.12	-3.47	-4.66	-4.92	-5.47		
276	7.91	A.	8			64				5	4	13	3	20	+0.49	-1.64	-5.77	-7.16	-7.56	-7.86		
277		D.	11	14	45	59				(17)	5	5	2	12	+0.48	-0.43	-1.55	-5.88	-6.26	-6.48		
278		D.																-6.8	-7.64	-9.11	-10.44	
279		D.																-8.89	-9.9	-10.44		
280		D.																				
281		D.																				
282		D.																				
283		D.																				
284		D.																				
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379																						

* Supernatant, sodium phosphate buffer, $\mu = 0.3$.

† A., ascending; D., descending.

‡ Additional component present (γ_3 , 30 per cent; $u = -1.51 \times 10^{-5}$).

mobility, there was invariably observed an appreciable β fraction of intermediate charge. In the majority of cases, the α fraction was resolved into three individual components, migrating with sharply defined, needle-like peaks. The reproducibility of the phenomenon was such as to leave little room for the assumption that these boundaries are artifacts or "false boundaries" due to overheating and convection processes in the cell. The γ fraction was also clearly made up of several components of a closely similar mobility. In three experiments, the β fraction was resolved into three individual components. Finally, there was almost always present in the anode limb of the cell a well defined δ -boundary with a measurable cathodic mobility which displayed a tendency to decrease when the pH was raised. Up to eight different components could be distinguished in some diagrams obtained in this series in spite of the fact that the total

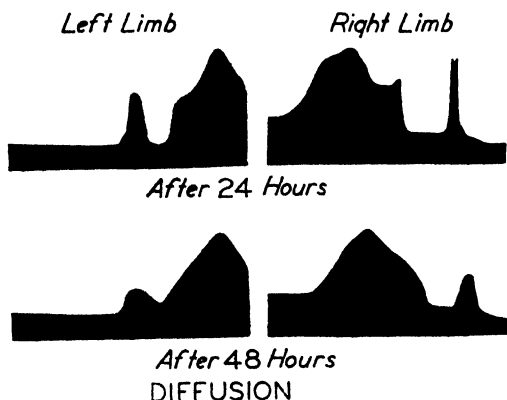


FIG. 3. Diffusion diagrams of the system shown in Fig. 2, recorded 24 and 48 hours respectively after the current source was disconnected.

protein concentration could not be greater and was probably much lower than 1.2 per cent, since a large fraction of the nitrogen was in dialyzable form. The absolute concentration of some of the α or β components must have been of the order of 0.01 per cent, since their relative concentration, as computed from the area under these peaks, amounted to only a few per cent of the total mass of material responsible for the refractive index gradients in the cell. This illustrates the sensitivity of the schlieren method and the possibility of detecting trace components in the presence of substantial amounts of other materials if the conditions for electrophoretic resolution are favorable. Direct visual observations of the material in the cell during the run revealed in some instances an unusually sharp and slightly cusped appearance of the boundaries, indicative of slight boundary anomalies. These anomalies were observed, although the amount of current flowing

through the cell was not excessive (potential gradients from 1.5 to 3.7 volts per cm.) and although the conductivity and pH of the yeast extract

TABLE III—*Electrophoretic Analyses of Macera-*

Ex- peri- ment No.	Dried yeast pre- paration* and batch No.	pH†	Bound- aries	Relative concentrations of individual components, per cent												
				δ	γ_1	γ_2	γ_{total}	β_1	β_2	β_3	β_{total}	α_1	α_2	α_{total}		
275	Hull (2)	7.91	A.	10	35	42	77					8	3	2	5	
	Undialyzed		D.	23			66	5	3			8	1	2	3	
261	Hull (2)	7.7	A.		32	60	92					6			2	
	Dialyzed		D.		14	71	85					8			7	
276	Hull (2c)	7.9	A.		56	30	86					7			7	
	Undialyzed		D.		27	59	86		$\alpha + \beta = 14$							
283	Hull (2c)	7.72	A.		38	56	94					4			2	
	Dialyzed		D.				85					8			7	
278	Federal (9)	7.78	A.	13			51	7	23	1	31	2	2		4	
	Undialyzed		D.		42	23	65	7	23		30				5	
280	Federal (9)	7.74	A. } D. }	1 component												
277	Krueger (10)	7.9	A.	10			33				57					
	Undialyzed		D.				30				70					
279	Krueger (10)	7.70	A.	16			73				10				1	
	Dialyzed		D.	8	35	41	76				12				4	
284†	Krueger (10)	7.2	A.				93	6	2		8					
	Undialyzed		D.	8			82	7	3		10					
281	Krueger (11)	7.97	A.				34				66					
			"	9			40	27	21	3	51					
	Undialyzed		D.				38				62					
			"				45	24	31		55					
282	Krueger (11)	7.71	A.	21			70				9					
	Dialyzed		D.	5				80			9				4	
285	Krueger (12)	7.80	A.	39							51				14	
			"	33							45	10	6		16	
	Undialyzed		D.‡				23				62	3	13		16	
			D.				32				55	5	5		10	
286	Krueger (12)	7.75	A.	6			16				69	8	3		11	
	Dialyzed		D.	Diagram not suitable for measurement												

* Hull, Hull's top yeast; Federal, Federal bakers' yeast; Krueger, Krueger's

† Solvent, 0.1 sodium phosphate buffer, $\mu = 0.3$ (except in Experiment 284).

‡ 0.167 M diammonium phosphate used for extracting yeast and as supernatant

§ Additional minor component (1.5 per cent; $u = -5.47 \times 10^{-4}$) present.

had been made closely similar to those of the supernatant buffer prior to the run. A possible explanation for this observation might reside in differences in the *qualitative* composition of the inorganic buffer ion systems pres-

ent in the yeast extract and in the supernatant fluid. It must be borne in mind, however, that maceration extracts of yeast contain phosphate ions

tion Extracts of Various Dried Yeast Preparations

Electrophoretic mobility, cm. ² sec. ⁻¹ volt ⁻¹ × 10 ⁶											Electro-phoresis period
δ	γ_1	γ_2	γ_{total}	β_1	β_2	β_3	β_{total}	α_1	α_2	α_{total}	
+0.75	-0.09	-2.29					-4.19	-4.65	-5.14		
+0.35			-1.54	-3.92	-4.21	-4.29		-4.67	-5.00		
	-0.68	-3.34					-5.98			-11.79	
	-0.65	-2.52					-5.10			-11.13	
	0	-2.4					-4.12	-5.14	-5.25		
	+0.39	-0.82					-2.49	-3.04	-3.46		
	+0.58	-1.96					-4.38			-7.01	
			-1.76				-3.45			-7.75	
+0.95			+0.29	-0.99	-1.61	-1.99		-2.57	-3.45		
	+0.29	-0.54		-1.02	-1.31			-1.79	-1.92		
			-0.78								
			-1.19								
+0.74			0				-1.61				
			+0.33				-1.42				
+0.17			-1.79				-3.87			-8.99	
(+0.42)			-1.59				-5.99			-14.57	
			-1.26	-4.76	-5.73						
+1.51			-1.81	-2.72	-3.20						
			+0.33				-2.09				1st
+1.46			0	-2.02	-2.35	-2.50					2nd
			+0.12	-1.42	-1.69						1st
			+0.14	-0.83	-1.94	-2.09					2nd
+0.05			-2.01				-3.89				
-0.43			-1.91				-3.93			-8.63	
+0.20			0				-2.11			-3.45	1st
+0.21							-1.89	-3.39	-3.6		2nd
			-0.62				-1.90	-3.65	-3.75		1st
			-0.54				-1.68	-3.83	-4.41		2nd
+0.66			0				-2.13	-3.78	-4.53		

bottom yeast.

for experiment.

in concentrations of the order of 0.1 M and that a phosphate buffer mixture was employed as the supernatant solution. Soon after the current was switched off, the boundaries assumed a normal appearance. The results

of the analysis of the diagrams recorded in this series of experiments on undialyzed maceration extracts from Hull's bottom yeast are compiled in Table II.

In the case of globular proteins endowed with a fair degree of symmetry, it is possible to gain information on their molecular dimensions by observing the rate of their free diffusion into a layer of supernatant solvent. Such

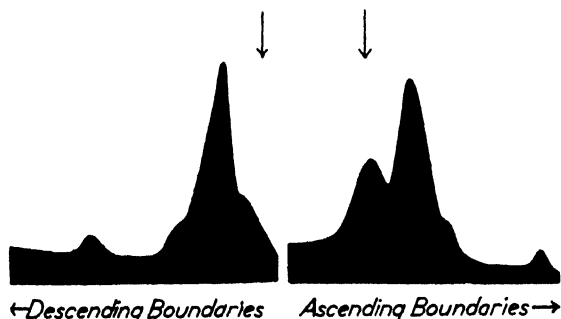


FIG. 4. Electrophoretic diagram of dialyzed maceration extract of dried, Hull's top yeast, recorded after 245 minutes electrolysis at pH 7.70. Potential gradient, 2.4 volts per cm.; nitrogen content, 2.0 mg. per cc.

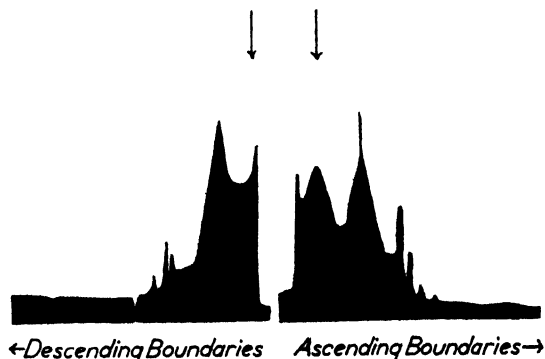


FIG. 5. Electrophoretic diagram of undialyzed, diluted maceration extract of dried, Hull's top yeast, recorded after 315 minutes electrolysis at pH 7.91. Potential gradient, 2.4 volts per cm.; nitrogen content, 2.6 mg. per cc.

observations may be made on boundaries arising during electrophoretic separation of complex mixtures simply by following the rate of their spreading with the schlieren scanning method after the current has been disconnected.¹ Diffusion diagrams of this type were frequently recorded in

¹ Longworth (6) and Rothen (7) have employed the Tiselius electrophoresis cell and the schlieren method for the measurement of diffusion rates of single proteins without preceding electrophoresis.

the course of this work. Representative examples are reproduced in Fig. 3. It can be seen that the boundaries of the various components contained in these extracts spread at different rates, thus indicating considerable differences in molecular weight. The true molecular weights of these proteins may be determined independently of the factor of shape by combining, in

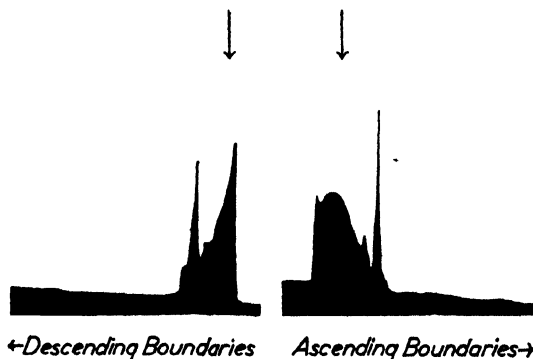


FIG. 6. Electrophoretic diagram of undialyzed, diluted maceration extract of dried, Federal bakers' yeast, recorded after 387 minutes electrolysis at pH 7.78. Potential gradient, 2.4 volts per cm.; nitrogen content, 1.87 mg. per cc.

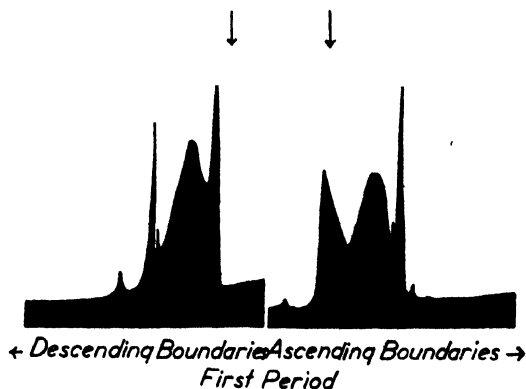


FIG. 7. Electrophoretic diagram of undialyzed, diluted maceration extract of dried, Krueger's bottom yeast, recorded after 367 minutes electrolysis at pH 7.80. Potential gradient, 2.26 volts per cm.; nitrogen content 2.5 mg. per cc.

the well known equation, given by Svedberg, the diffusion coefficients obtained in this manner with their sedimentation constants which remain to be determined.

Following the study of the electrophoretic behavior of maceration extracts obtained from Hull's bottom yeast, a series of thirteen experiments was conducted on dialyzed and undialyzed Lebedev extracts prepared from

other yeast preparations; viz., *Hull's top yeast*, *Federal bakers' yeast*, and three different batches of dried *Krueger's bottom yeast*. Typical diagrams obtained in these experiments are reproduced in Figs. 4 to 9. The results of these experiments are presented in Table III.

In order to obtain comparable results in this survey of various yeast preparations, 0.1 M sodium phosphate buffer of pH 7.8 (ionic strength, 0.3)

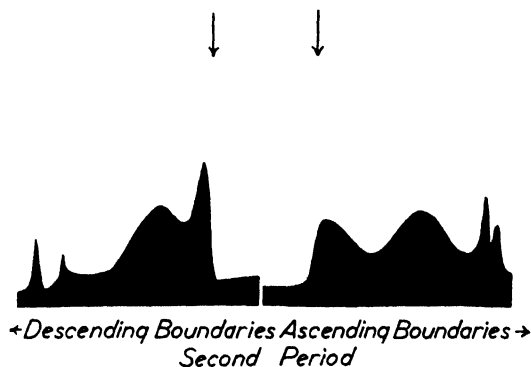


FIG. 8. Diagram of system shown in Fig. 7, recorded after electrolysis was continued for an additional 701 minutes at a potential gradient of 1.23 volts per cm.

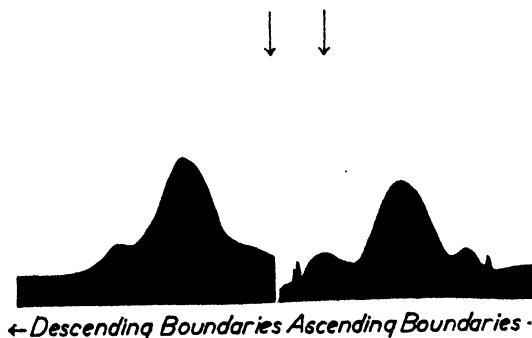


FIG. 9. Electrophoretic diagram of dialyzed maceration extract of dried, *Krueger's bottom yeast*, recorded after 625 minutes electrolysis at pH 7.75. Potential gradient, 2.27 volts per cm.; nitrogen content, 2.6 mg. per cc.

was employed as the supernatant solution with a single exception. In most instances the electrophoretic patterns obtained were qualitatively rather than quantitatively similar to those secured with extracts of *Hull's bottom yeast*. Again, the diagrams obtained with dialyzed extracts were less complex than those of undialyzed extracts. Again, three different groups of components (labeled α , β , and γ fractions) were found, but the extent of resolution varied and appreciable differences existed between the

various systems with respect to the mobility and relative concentrations of the individual components. The results obtainable with extracts prepared from the same batch of dried yeast and from different batches of yeast of the same type and provenience are fairly reproducible, provided that the conditions of drying and of preparing the maceration extracts are identical. The importance of the solvent employed in the extraction process is emphasized by the striking differences in the electrophoretic diagrams of the undialyzed maceration extracts of Krueger's bottom yeast (Batch 10) prepared with tap water and diammonium phosphate respectively (Experiments 277 and 284, Table III). The latter extract fermented glucose and fructose actively, in contrast to the former. However, it is improbable that the mere state of activity of the zymase complex in these solutions could influence so profoundly the electrophoretic pattern in which all soluble yeast colloids are represented. It should be mentioned that the aqueous extract was examined at a nitrogen concentration about 4 times lower than that of the ammonium phosphate extract (1.9 and 7.7 mg. of N per cc. respectively).

DISCUSSION

The quantitative study of the behavior of unfractionated maceration extracts of dried yeast (Lebedev extracts) in an electrical field with the aid of an integrating refractive index method has demonstrated the presence of a number of electrochemically different components. In addition to their electrophoretic mobility, some information was gained concerning their relative concentrations on the assumption that the various components possess comparable molar refractive increments. The solutions were sufficiently dilute to exclude appreciable mutual hindrance during the migration of the individual components in the electrical field. The complexity of the system under study is readily understandable, since extracts prepared in this manner are known to contain, in addition to "inert" soluble proteins and polysaccharide (yeast gum) derived from the yeast cell, a large number of enzymes, *e.g.* proteinase, dipeptidase, catalase, flavoproteins, and the complete enzyme system of fermentation (zymase complex). Special dehydrogenases, such as the lactic acid enzyme, and hemochromogens are also present. The present investigation confirms and greatly extends the observations made several years ago by the writer in collaboration with J. L. Melnick, in the course of studies on yeast carboxylase (8). The qualitative examination of various yeast extracts in the Tiselius apparatus with the aid of the simple Foucault-Toepler schlieren method indicated at that time the presence, in such extracts, of three groups of colloids distinguishable by their electrophoretic behavior at pH 7.2. Indications were also obtained that the carboxylase migrates with the group of colloids of

intermediate electrical mobility, although the boundary of the enzyme is in all probability not visible as a separate entity in crude yeast extracts because of its low concentration.

The observation made in the present work, *viz.* that the electrophoretic diagrams obtained with *undialyzed*, equilibrated yeast extracts are more complex than those given by the *dialyzed* solutions, might possibly be explained by the removal of certain low molecular substances (coenzymes, metal ions, etc.) in the course of the dialysis. These substances exist in the native extracts, at least partially, in loose, dissociable combination with specific proteins. Thus, cozymase combines with active proteins to form pyridinoproteins; *e.g.*, alcohol, glyceraldehyde phosphate, and glycerophosphate dehydrogenase. Adenylic acid is considered to be the prosthetic group of enzymes active in transphosphorylation reactions, cocarboxylase and magnesium are components of carboxylase, while magnesium is a constituent of enolase. Yeast aldolase, according to recent work of Warburg and Christian (9), contains heavy metal, probably ferrous iron, and a function of manganese in fermentation also appears probable. It is conceivable that after the removal of these low molecular components by dialysis the protein carriers remaining in the non-dialyzable fraction possess a charge distribution differing from that of the complete enzyme systems and become less differentiated in their electrochemical characteristics. In this manner a pool of chemically similar proteins may be formed, migrating as a unit in the electrical field. A counterpart of this phenomenon might perhaps be found in the production of electrochemically distinct, immune proteins (antibodies) from the reservoir of "unspecific" serum globulins by immunization of the organism with specific antigens. The differences observed here between the electrophoretic patterns given by extracts of different yeasts are not much larger than those existing between the patterns of the blood sera of different animal species. Little is known about the electrophoretic behavior of individual yeast enzymes beyond the non-optical experiments on Warburg's "old" yellow enzyme by Theorell (10) and on crude hexokinase preparations by Meyerhof and Möhle (11). The logical extension of the present work consists in the study of the electrophoretic properties of isolated yeast enzymes by optical methods.

SUMMARY

The electrophoresis of unfractionated maceration extracts (Lebedev juice) derived from various dried yeast preparations has been studied in the Tiselius apparatus, by means of an integrating refractive index method.

A number of electrochemically different colloidal components were observed in these extracts and their mobilities, as well as their relative concentrations, were determined.

The electrophoretic patterns of undialyzed yeast extracts are more complex than those of the dialyzed solutions. This phenomenon is linked to the removal of low molecular components, *e.g.* coenzymes and metal ions, by the dialysis of yeast extracts.

Marked quantitative differences were found in the diagrams obtained with maceration extracts of different yeast types.

The author wishes to acknowledge, with appreciation, the technical assistance rendered by Mr. Charles Lawrence and Miss Helen Trief. This work forms a part of a research project on glycerol production by yeast fermentation, conducted under the auspices of the Food Distribution Administration of the United States Department of Agriculture. Its progress was greatly aided by the stimulating interest shown by Mr. James S. Wallerstein of this Foundation.

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Note Added in Proof—Celloseal, which was used in the present experiments as the lubricant for the Tiselius electrophoresis cell, should not be employed in experiments with blood serum or plasma, since it gives rise to anomalies in the electrophoretic pattern of the ascending boundaries. Yeast extracts apparently do not possess the solvent action on this grease exhibited by serum proteins.

THE INTERFERENCE OF SESAME OIL, FISH OIL, AND CHOLESTEROL WITH THE POLAROGRAPHIC DETERMINATION OF α -TOCOPHEROL*

BY J. J. BEAVER AND HANS KAUNITZ

(From the Department of Chemistry, and the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, October 27, 1943)

In view of the physiological importance of tocopherols, repeated attempts have been made to determine them quantitatively in animal tissues. Several authors (1-4) have reported methods for the determination of tocopherol in tissue extracts and have noted the difficulties caused by substances extracted along with tocopherols, such as fat, cholesterol, and carotenes.

In the method of Smith, Spillane, and Kolthoff (5), the polarograph was used for quantitative measurements of pure α -tocopherol in 75 per cent alcohol in the presence of an acetate buffer. These experiments were repeated with the electropode of the Fisher Scientific Company (6). The curves obtained with α -tocopherol under conditions similar to those described by Smith *et al.* (5) showed a definite half-way potential at approximately +0.25 volt at 31° compared with the reported value of +0.28 volt at 25°; and further, the i_d is proportional to the concentration of tocopherol. Therefore, the quantitative determination of pure α -tocopherol is possible.

Since tocopherols in tissue extracts are always associated with fats and cholesterol, the next problem was to test the method in the presence of such substances. Fats in concentrations of 2 per cent were found to be insoluble in the acetate buffer and in a citrate buffer of pH 4.6, when 75 per cent alcohol was used as a solvent. Attempts to obtain polarographic curves by emulsifying the fats with 0.1 per cent of a spreading reagent (Arescap) failed completely.

Finally a buffer was prepared containing 0.025 M sodium benzoate and 0.025 M benzoic acid in 20 cc. of acetone and 80 cc. of water; 2.25 cc. of this buffer, 5 cc. of fat, and 92.75 cc. of acetone gave a clear solution. The currents obtained with the polarograph were approximately 0.1 per cent of those found with the acetate buffer in 75 per cent alcohol. With the sensitivity of the instrument used in these measurements (0.018 microampere per division), the differences in the current at the half-way potential (about +0.35 volt, Fig. 1) between the buffer curve and those for the mixture were nearly of the same magnitude as the error involved. While it was possible to obtain readings with a 10^{-3} M α -tocopherol concentration in the presence

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of 2.5 to 5 per cent of sesame oil or fish oil (Mead's, blended) or in 0.15 per cent cholesterol, the small currents (maximum 0.5 to 1 microampere) and the variability of the readings made the quantitative determination of α -tocopherol extremely inaccurate.

As can be seen from Fig. 1, the sesame oil exerts a depressing effect on the current roughly proportional to its concentration. The effect of the 2.5 per cent solution is equivalent to the effect of an approximately 10^{-3} M α -tocopherol solution (Curves II and III). The influence of the fish oil used was

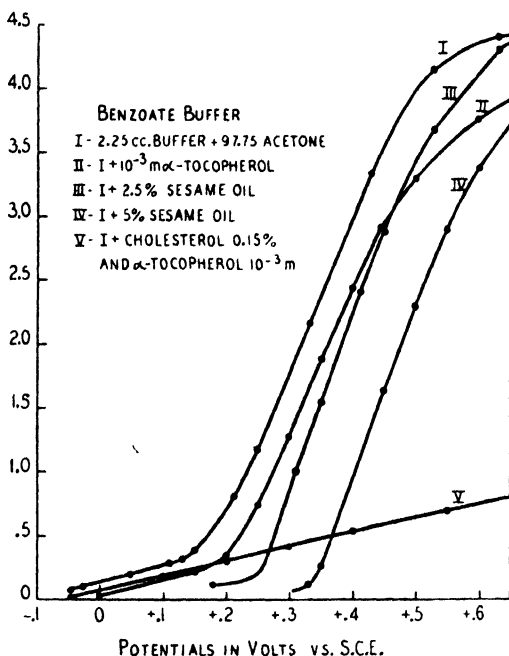


Fig. 1. α -Tocopherol, sesame oil, and cholesterol in benzoate buffer. The ordinate scale is given in microamperes.

similar, while the effect of 0.15 per cent cholesterol was even more pronounced (Curve V).

SUMMARY

1. The method of Smith, Spillane, and Kolthoff for the quantitative polarographic determination of a solution of pure α -tocopherol has been confirmed.

2. Sesame oil, fish oil (Mead's, blended), and cholesterol depress the polarographic curve roughly in proportion to their concentration in the buffer solution.

3. The above effect, coupled with the inaccuracy of the readings under the given conditions, prevents the quantitative determination of α -tocopherol in concentrations of 10^{-3} M in solutions containing 2.5 to 5 per cent of the oils or 0.15 per cent cholesterol.

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NOTE ON PLASMA FIBRINOGEN IN GUINEA PIG SCURVY*

By WILLIAM R. SULLIVAN, EDWARD O. GANGSTAD,
AND KARL PAUL LINK

(From the Department of Biochemistry, Wisconsin Agricultural Experiment Station,
University of Wisconsin, Madison)

(Received for publication, November 22, 1943)

In a recent paper we reported that total and partial depletion of vitamin C in the guinea pig produced no change in the prothrombin level (or activity) of 12.5 per cent plasma and that adequate or high intakes of *l*-ascorbic acid did not affect the prothrombin time (1). In conjunction with this work the effect of the vitamin on the fibrinogen content of plasma in normal and scorbutic guinea pigs was also studied.

Relatively few quantitative data have been published on fibrinogen in experimental scurvy ((2) p. 325). Ohata (3) determined the fibrinogen content with a refractometric method and concluded that it decreased slightly after the frank symptoms of scurvy developed. We could not confirm this observation. Our findings, based on a chemical method, indicate that the fibrinogen content increases with the onset of scurvy and that feeding either raw cabbage or *l*-ascorbic acid along with the basal diet restores the normal level within 2 weeks.

EXPERIMENTAL

The essential details of the animal experiments have already been described (1). The guinea pigs used were those represented in the preceding article ((1) Fig. 1, p. 479). Fibrinogen was determined by the method of Folin and Ciocalteau (4) as modified by Reiner (5) on an aliquot of the plasma used for the prothrombin estimations. The 82 guinea pigs used were first maintained for 2 weeks on the scorbutogenic diet supplemented with 40 to 50 gm. of raw cabbage daily. During this interval the normal range of fibrinogen content was determined. The cabbage was then omitted and the following supplements were fed: Group A, no supplement; Group B, 0.5 mg. of *l*-ascorbic acid per day; Group C, 2 mg. of *l*-ascorbic acid per day; Group D, 25 mg. of *l*-ascorbic acid per day; and Group E, 40 to 50 gm. of raw cabbage per day. Blood samples were taken at 7 day intervals.

The results are summarized in Fig. 1. Each point on the curves repre-

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sents an average value for groups of six or more guinea pigs selected on the basis of uniformity at the beginning of the experiment.

The fibrinogen content of normal guinea pig plasma ranged from 205 to 245 mg. per 100 cc. of plasma, and essentially this same range was observed in guinea pigs fed the scorbutogenic ration but protected against scurvy by daily supplements of 40 to 50 gm. of raw cabbage or 2 mg. of *l*-ascorbic acid (Fig. 1, Curves C and E). The guinea pigs receiving the large quantity of the vitamin (25 mg. per day) also showed normal fibrinogen values.

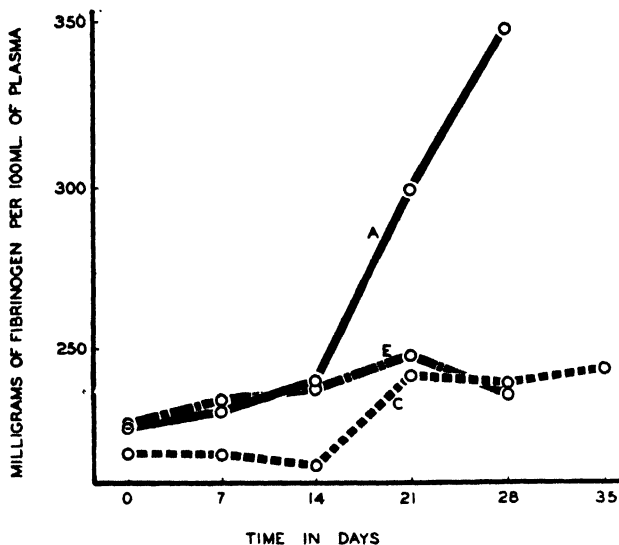


FIG. 1. The effect of *l*-ascorbic acid intake on the fibrinogen content of guinea pig plasma. Curve A represents plasma from guinea pigs receiving no *l*-ascorbic acid, Curve C, from those receiving 2 mg. of *l*-ascorbic acid daily, and Curve E, those receiving 40 to 50 gm. of raw cabbage per day.

In contrast the plasma of the guinea pigs on the unsupplemented scorbutogenic ration showed a marked increase in fibrinogen content after the 2nd week, when the usual symptoms of scurvy became evident. After 4 weeks the plasma of these animals contained 350 mg. of fibrinogen per 100 ml. (Fig. 1, Curve A). An increase in fibrinogen was also observed in another group of guinea pigs that was partially protected against scurvy by the supplemental feeding of 0.5 mg. of *l*-ascorbic acid per day, the level being 305 mg. per 100 ml. of plasma in the 5th week of the experiment.

Observations on the restoration of normal fibrinogen levels in scorbutic guinea pigs were also made. After 4 weeks, the animals of Group A, with

fibrinogen in the range of 340 to 350 mg. per 100 ml. of plasma, were fed either 40 to 50 gm. of raw cabbage or 2.0 mg. of *l*-ascorbic acid per day. The daily feeding of the supplement usually restored the fibrinogen level to the normal range by the 6th week (2 weeks of curative treatment).

SUMMARY

The fibrinogen content of guinea pig plasma increases markedly with the onset of scurvy. Restoration of normal fibrinogen levels in scorbutic guinea pigs follows within 2 weeks after the basal diet is supplemented either with raw cabbage or *l*-ascorbic acid.

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ADENOSINE TRIPHOSPHATE IN MUSCLES OF RATS STUDIED WITH RADIOACTIVE PHOSPHORUS*

By EUNICE V. FLOCK AND JESSE L. BOLLMAN

(From the Division of Experimental Medicine, The Mayo Foundation, Rochester, Minnesota)

(Received for publication, October 29, 1943)

Immediate decreases of the concentrations of glycogen and phosphocreatine are found in continuously working muscles, and many seconds later decreases of the adenosine triphosphate are found also. The hydrolysis of the adenosine triphosphate, which involves the liberation of 2 moles of phosphate, may be detected in 40 seconds and reaches its maximum in 1 or 2 minutes in muscles working at the rate of three contractions per second (1). The concentration of adenosine triphosphate remains low as work continues and is restored to its original level slowly when rest ensues.

Hydrolysis and resynthesis of adenosine triphosphate without an appreciable change of its concentration are continuously occurring in resting muscles, according to studies with radioactive phosphorus. The uptake of P^{32} by the acid-labile phosphate groups is fairly rapid, as shown by Hevesy and Rebbe (2, 3) and by Sacks and Altschuler (4, 5), while the uptake by the stable phosphate group is extremely slow, as shown by Korzybski and Parnas (6). Sacks did not find any change in the distribution of P^{32} in adenosine triphosphate from a 15 second period of isometric contraction.

Lohmann (7) showed that the dephosphorylation of adenosine triphosphate is a stepwise process involving the formation of adenosine diphosphate and then of adenylic acid. The first step is catalyzed by the enzyme adenosine triphosphatase which Engelhardt and Ljubimowa (8) and others (9) have shown to be myosin; the second step is catalyzed by a more soluble enzyme, adenosine diphosphatase.

We have measured the uptake of P^{32} by each of the phosphate groups in the adenosine triphosphate of muscles which were resting, working, or recovering from work. The experimental procedure involved the isolation of adenosine triphosphate, hydrolysis with myosin, and isolation of adenosine diphosphate, then hydrolysis with acid. The phosphorus content and its radioactivity for each mg. of phosphorus of each fraction hydrolyzed furnished the basis of comparison. The P^{32} of the adenosine triphosphate from resting muscle at the end of an hour was distributed in a proportion

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of approximately 100 for the first phosphate group, hydrolyzable with myosin, 75 for the second, hydrolyzed from adenosine diphosphate by acid, and 5 for the third phosphate group, which remains as ribose phosphate. The P^{32} content of adenosine triphosphate of muscle contracting 180 times each minute for 1 hour was increased somewhat more than that of the corresponding resting muscle in the same animal. This increase of radioactivity was less than that found when resynthesis of about three-fourths of the adenosine triphosphate was accomplished in the same time by three periods of work followed by rest.

EXPERIMENTAL

Male rats, weighing about 240 gm., were anesthetized with pentobarbital sodium and injected intravenously with from 0.4 to 2.2 mg. of phosphorus as dibasic sodium phosphate containing from 20 to 80 microcuries of radioactive phosphorus. We are greatly indebted to Professor John H. Lawrence and Dr. Carl Helmholtz, of the Radiation Laboratory of the University of California, who have supplied the radioactive sodium phosphate used in these studies. In studies of the effect of work the Achilles tendon of the left leg of the rat was loaded with a 100 gm. weight and stimulated directly to contract three times a second for a specific period, as previously described (1). At the desired period the muscles of the left leg were quickly removed, frozen with dry ice and alcohol, weighed, and extracted with cold trichloroacetic acid, as previously described. The muscles of the right, unstimulated leg were similarly treated and used as a basis of comparison. A specimen of plasma was also extracted with trichloroacetic acid.

Adenosine triphosphate was isolated by precipitation from the trichloroacetic acid extract after neutralization to methyl red as the mercury salt, followed by two precipitations as the acid barium salt from 50 per cent alcohol, essentially as described by Kerr (10) (Fig. 1). The adenosine triphosphate thus obtained had a ratio of labile phosphorus to total phosphorus very close to $\frac{2}{3}$ and did not contain more than a trace of inorganic phosphate.

The method which was used to separate the three phosphate groups is shown in Fig. 2. The adenosine triphosphate was hydrolyzed with myosin prepared from rabbit muscle and used according to the methods of Ljubimowa and Pevsner (11). The myosin was always assayed against adenosine triphosphate of high purity prepared from dog muscle by the method of Kerr. The myosin was considered satisfactory when it did not contain either inorganic phosphate or diphosphatase. After the myosin hydrolysis both the adenosine diphosphate and Phosphate 1 were precipitated as barium salts and then separated by precipitation of the adenosine diphosphate as the mercury salt. After removal of mercury with hydrogen

sulfide the adenosine diphosphate was hydrolyzed with N sulfuric acid at 100° for 15 minutes. Phosphate 2 was precipitated as the magnesium ammonium salt. The quantity of phosphorus in each phosphate group was determined photelometrically by the method of Fiske and Subbarow (12). The radioactivity of each group was measured by a scale-of-four Geiger counter of the immersion type (13) and counts per second per mg.

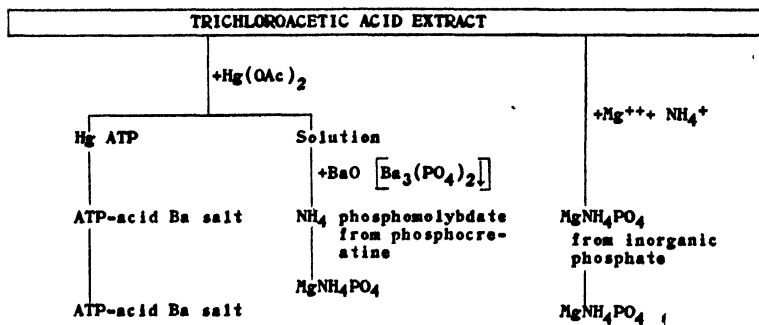


FIG. 1. Isolation of adenosine triphosphate from trichloroacetic acid extract of muscle.

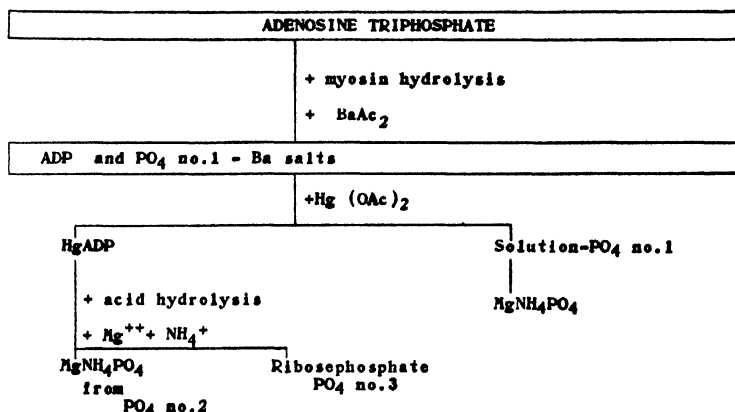


FIG. 2. Method of separation of the three phosphate groups

of phosphorus were calculated. It has been impossible to prevent some overlapping of these fractions with the relatively small amounts of muscle used for analysis. Such errors tend to minimize the differences in the radioactivities of the three phosphate groups but do not obscure them. The total radioactivity of the isolated adenosine triphosphate as calculated from the average of the three groups was in each case very close to that found by direct determination before hydrolysis.

The inorganic phosphate of the muscle was precipitated directly from the trichloroacetic acid extract as the magnesium ammonium salt, reprecipitated, and then analyzed for P and P^{32} content (Figs. 1 and 2).

TABLE I

Distribution of P^{32} in Adenosine Triphosphate in Resting Muscles

Radioactivity is expressed as counts per second for each mg. of phosphorus. The muscles of the hind legs were taken for analysis the indicated number of minutes after intravenous injection of dibasic sodium phosphate containing P^{32} as indicated. The muscles of two or four similarly treated rats were pooled for analysis.

P^{32}		Inorganic P	Adenosine triphosphate			Phosphocreatine
<i>microcuries</i>	<i>min.</i>		Phosphate 1	Phosphate 2	Phosphate 3	
20	2	534	62	27	0	47
20	2	1144	116	22	1	72
20	5	954	85	17	2	45
20	15	375	58	32	0	47
80	15	1435	334	179	11	
80	15	2795	408	165	40	
40	30	760	143	83	4	92
20	60	268	60	54	6	
20	60	277	79	63	4	
20	60	256	60	48	5	
20	60	331	68	54	5	
30	60	494	103	70	5	84
80	60	924	310	263		
80	60	1650	153	120	8	
80	60		195	120	12	
80	60	803	297	215	12	209
80	60	1151	444	327	27	
26	1440	286	213	197		242

Results

Resting Muscles—In different experiments 20 to 80 microcuries of P^{32} were administered to rats and from 2 to 60 minutes later the resting muscles of the hind legs were removed and four or eight pooled for analysis. The radioactivity of the adenosine triphosphate per mg. of phosphorus was much lower than that of the inorganic phosphate during the 1st hour after the administration of radioactive phosphorus. Of the three phosphate groups in the adenosine triphosphate, Phosphate 1 had the greatest radioactivity during this period and Phosphate 3 the least (Table I). If the

radioactivity of each Phosphate 1 was arbitrarily made 100, then the average value for Phosphate 2 was 75 and for Phosphate 3 it was 5 at the end of the hour. Lower values than 75 for Phosphate 2 were consistently found during the first part of the hour. Thus P^{32} enters Phosphate 1 at the greatest rate, but with the lapse of time the radioactivity of Phosphate 2 tends to approach the same value. In one experiment resting muscles were analyzed 24 hours after the administration of P^{32} , at which time the radio-

TABLE II

Effect of Work on Distribution of P^{32} in Adenosine Triphosphate

Radioactivity is expressed as counts per second for each mg. of phosphorus. 90 minutes elapsed between the time of injection of P^{32} and the taking of muscles in all experiments except Nos. 10 and 17, in which 60 minutes elapsed. The indicated period of work immediately preceded taking the specimens of muscle.

	Experiment No.	Inorganic phosphate		Adenosine triphosphate					
		Left	Right	Total phosphate		Acid-labile phosphate		Stable phosphate	
				Left	Right	Left	Right	Left	Right
Controls, no work	1	231	187	67	61				
	2	303	289	117	91				
	3	404	524			230	198	6	11
	4	826	956	154	148	238	266	39	49
Muscles of left leg worked 1 min.	5	566	896	308	285				
	6	530	746	232	190	323	293	40	21
	7	479	682			320	308	60	40
	8	654	1227			332	281	19	16
	9	826	1310	264	251	486	404	23	12
	10	661	1124	216	224	358	324	36	38
Muscles of left leg worked 60 min.	11	433	849			258	186	13	3
	12	560	1099	194	177	270	213		
	13	716	983	222	188	372	301	63	30
	14	717	1037			377	333	33	15
	15	793	1030	285	214	485	376	29	10
	16	602	801	264	188	433	333	105	68
	17	884	1380	259	258	420	387	23	26

activities of Phosphates 1 and 2 were approximately the same. The radioactivity of the phosphocreatine was lower than that of Phosphate 1 of the adenosine triphosphate in each of seven experiments in which it was measured.

Effect of Work—In the first series of experiments on working muscles 80 microcuries of radiophosphorus were administered to each rat. The muscles of the left hind leg were stimulated to work for 1 or 60 minutes. The working muscles of two rats were combined for analysis, as were the control

resting muscles of the right hind legs. The adenosine triphosphate was hydrolyzed with acid and the acid-labile phosphate separated from the stable ribose phosphate by precipitation as the magnesium ammonium salt. The mean difference between the labile phosphate of the five muscles which worked 1 minute and that of the control resting muscles was 42 ± 12 counts

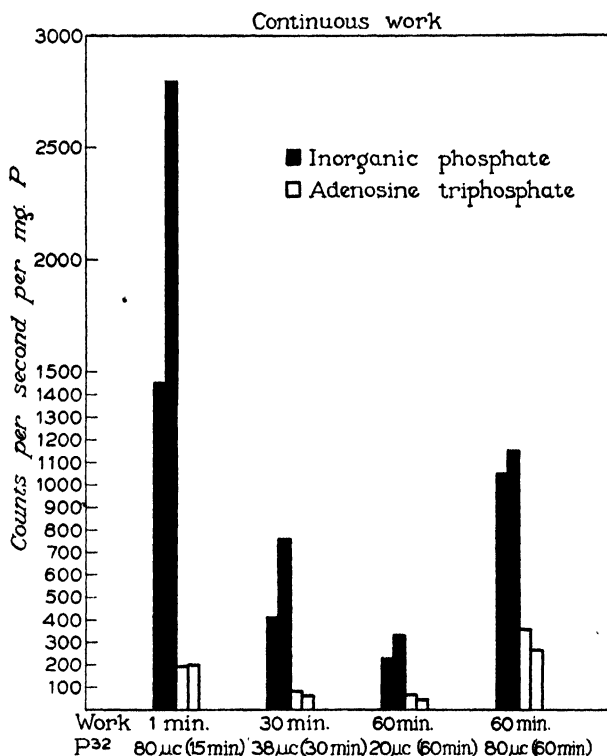


FIG. 3. The first bar in each group indicates the radioactivity of the inorganic phosphate of the combined working muscles of that experiment. The second bar indicates that of the corresponding resting muscles. The values for radioactivity of the isolated adenosine triphosphate are similarly shown. The period of work was coincident with the time after injection of P^{32} , except that the 1 minute work period was the terminal minute of the first experiment.

per second for each mg. of phosphorus, which is of questionable significance; the mean difference between the seven muscles which worked 60 minutes and the control muscles was 69 ± 10 counts per second, which is statistically significant (Table II). These figures indicate an increased radioactivity of 13 per cent with 1 minute of work and only a subsequent 10 per cent increase during 59 additional minutes of work. The radioactivity

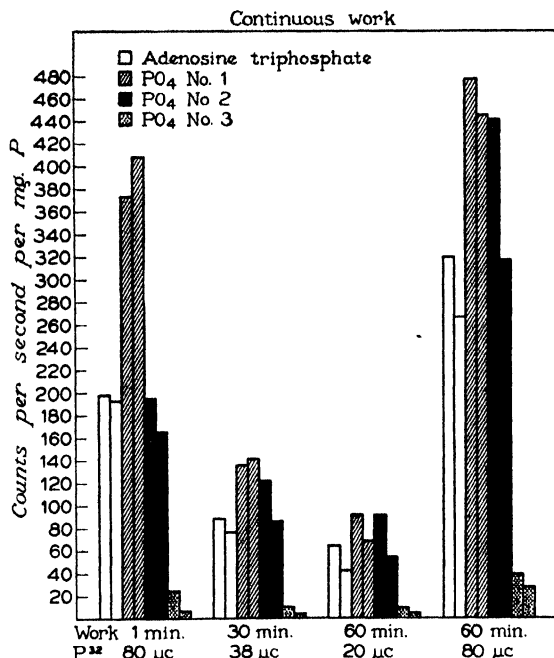


FIG. 4. The values for radioactivity of the isolated adenosine triphosphate from working and resting muscle are repeated from Fig. 3 on a larger scale with the values for each phosphate group obtained after fractionation.

TABLE III

Resynthesis of Adenosine Triphosphate Following 5 Minute Period of Work

Mean concentrations of phosphorus are expressed as mg. per 100 gm. of muscle.

The muscles of the right legs rested throughout the experiment; the muscles of the left legs rested for the number of minutes specified after the 5 minute work period. The muscles of each rat were analyzed separately.

No. of rats	Time of rest min.	Inorganic P					Labile P of adenosine triphosphate				
		Left leg		Right leg		Mean difference*	Left leg		Right leg		Mean difference*
		Mean	S.D.	Mean	S.D.		Mean	S.D.	Mean	S.D.	
10	0	71.1	11.3	31.4	5.9	39.8 ± 4.1	16.8	4.5	39.2	2.7	-22.4 ± 1.5
12	2	54.7	18.7	32.7	4.3	22.0 ± 7.5	21.0	6.0	41.3	8.0	-20.2 ± 2.1
13	5	45.4	12.6	32.8	4.6	12.6 ± 3.5	19.9	4.6	37.8	4.2	-17.9 ± 1.8
9	10	33.5	6.0	33.5	4.2	0 ± 1.9	25.7	3.3	38.0	1.1	-12.3 ± 1.3
6	15	33.5	3.2	32.1	6.6	1.4 ± 1.6	27.4	4.2	39.5	4.3	-12.1 ± 1.5
8	25	31.2	2.7	31.8	5.0	-0.6 ± 1.4	25.8	2.8	36.8	2.2	-11.0 ± 1.4
12	55	24.2	6.9	29.4	5.2	-5.2 ± 1.7	29.9	4.8	32.9	4.3	-3.0 ± 0.8

S.D. indicates the standard deviation.

* The value following the $\pm$ sign is the standard error of the mean.

of the adenosine triphosphate after 1 minute of work was 58 per cent of that of the inorganic phosphorus and after 60 minutes of work it was 55 per cent. Since more than half of the adenosine triphosphate of muscle is hydrolyzed during 1 minute of work, the increase of radioactivity found in our experiments does not indicate any appreciable turnover of adenosine triphosphate in continuous work.

In the second series of experiments the muscles from four or eight hind legs were combined and the more extensive fractionation was attempted.

TABLE IV

Concentrations of Adenosine Triphosphate Following Alternate Periods of 5 Minutes of Work and 15 Minutes of Rest

Mean concentrations of phosphorus are expressed as mg. per 100 gm.

The muscles of the right legs rested throughout the experiment; the muscles of the left legs worked and rested for the number of minutes specified. The muscles of each rat were analyzed separately.

No. of rats	Time of work (W.) and rest (R.)	Inorganic P					Labile P of adenosine triphosphate				
		Left leg		Right leg		Mean difference*	Left leg		Right leg		Mean difference*
		Mean	s.d.	Mean	s.d.		Mean	s.d.	Mean	s.d.	
	<i>min.</i>										
10	5 W.	71.1	11.3	31.4	5.9	39.8 ± 4.1	16.8	4.5	39.2	2.7	-22.4 ± 1.5
6	5 " + 15 R.	33.5	3.2	32.1	6.6	1.4 ± 1.6	27.4	4.2	39.5	4.3	-12.1 ± 1.5
12	5 " + 15 " + 5 W.	77.0	7.4	32.8	2.9	44.2 ± 2.7	17.5	3.5	39.0	1.9	-21.5 ± 1.2
12†	2 (5 W. + 15 R.)	35.5	20.6	34.0	5.8	1.5 ± 6.1	26.9	7.5	39.2	3.3	-12.3 ± 2.3
6	2 (5 W. + 15 R.) + 5 W.	68.0	4.4	32.1	5.5	35.9 ± 2.2	21.9	2.4	39.3	5.9	-17.4 ± 2.2
8	3 (5 W. + 15 R.)	28.0	6.1	33.0	3.1	-5.0 ± 2.4	30.7	5.3	38.7	6.8	-8.0 ± 2.1

s.d. indicates the standard deviation.

* The value following the ± sign is the standard error of the mean.

† One rat in this group failed to show any recovery during the rest period.

From 20 to 80 microcuries of P^{32} were administered to each rat from 15 to 60 minutes before the muscles were removed (Fig. 3). The radioactivity of the inorganic phosphate of the resting muscles was greater with the larger amounts of P^{32} and the shorter intervals than with the smaller amounts of P^{32} and the longer intervals. The radioactivity of the inorganic phosphate of the plasma was usually 10 times as great as that of the inorganic phosphate of the muscle; the radioactivity of the latter was much greater than that of the adenosine triphosphate at the time intervals studied. The radioactivity of the inorganic phosphate was reduced in the

working muscles because of dilution with phosphate liberated from the less radioactive organic compounds. The radioactivity of the adenosine triphosphate showed a small increase in the working muscles. In both the working and the resting muscles the radioactivity of Phosphate 1 was higher than that of Phosphate 2 and this was much higher than that of Phosphate 3. The radioactivity of Phosphate 2 tended to approach that of Phosphate 1 in the working muscles (Fig. 4).

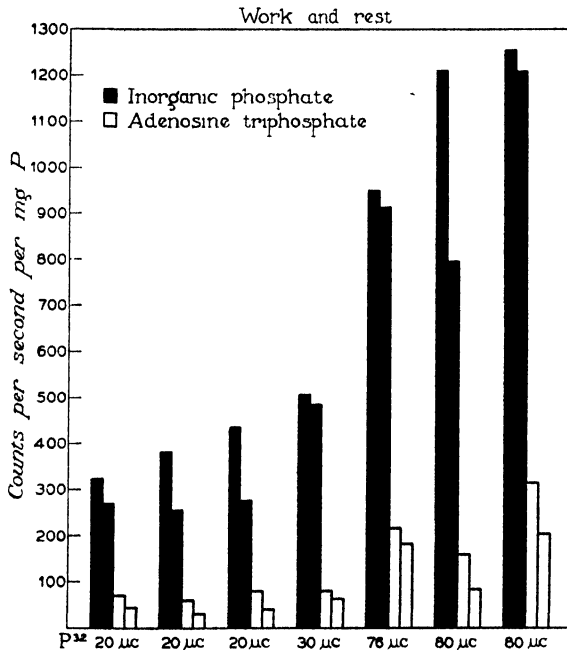


FIG. 5. The first bar in each group represents the radioactivity of the inorganic phosphate in the muscles of the left legs, which worked and rested for alternate periods of 5 and 15 minutes for 1 hour. The second bar indicates that of the corresponding resting muscles. The values for radioactivity of the isolated adenosine triphosphate are similarly shown. The first work period was begun in all cases immediately following the injection of P^{32} .

Effect of Work Followed by Rest—When the muscles of rats were stimulated to work for 5 minutes, a mean decrease of the concentration of adenosine triphosphate, expressed as mg. of labile phosphorus per 100 gm. of muscle, of 22.4 ± 1.5 was found, compared with the resting muscles (Table III). This difference was reduced to 12.3 ± 1.3 and 11.0 ± 1.4 mg. of phosphorus by a partial resynthesis during succeeding periods of 10 and 25 minutes of rest and to 3.0 ± 0.8 after 55 minutes of rest.

Groups of rats were studied after one or more alternating periods of 5

minutes of work and 15 minutes of rest. At the end of three such work periods there were mean increases in the concentrations of inorganic phosphate of 39.8, 44.2, and 35.9 mg. of phosphorus in the working muscles (Table IV); the increases practically disappeared during each rest period. Mean decreases in the concentration of the acid-labile phosphate of the adenosine triphosphate of 22.4, 21.5, and 17.4 mg. of phosphorus during each of the three work periods were found and these decreases were reduced to 12.1, 12.3, and 8.0 mg. respectively during the rest periods. Thus 10.3, 9.2, and 9.4 mg. of phosphorus were resynthesized to adenosine triphosphate during 1 hour. When such a turnover of adenosine triphosphate was pro-

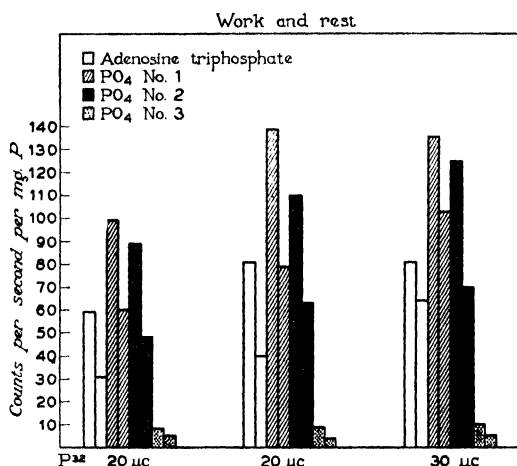


FIG. 6. The values for radioactivity of the isolated adenosine triphosphate from experimental and resting muscle from the second, third, and fourth experiments of Fig. 5 are repeated on a larger scale with values for each phosphate group obtained after fractionation.

duced in rats which had been injected with radioactive phosphorus, there was an increase of the radioactivities of both the inorganic phosphate and adenosine triphosphate of the stimulated muscle compared with the unstimulated muscle (Fig. 5). This increase was greater than that found after 60 minutes of continuous work. The increase of radioactivity of the adenosine triphosphate was found in both Phosphates 1 and 2 (Fig. 6).

Comment

The relatively slow exchange of P³² between the inorganic phosphate of plasma and muscles, which we have found, has been observed previously by Hevesy and Rebbe (2, 3), Sacks and Altschuler (4, 5), and others, as has the slower uptake of P³² in the adenosine triphosphate. The uptake of

P^{32} by the labile phosphate of adenosine triphosphate in muscle extracts has been shown, however, by Meyerhof (14) to be a much faster process.

While our studies confirm the observations of others that the uptake of P^{32} in the acid-labile phosphate groups in adenosine triphosphate is much greater than in the stable phosphate group, they show in addition that the uptake of P^{32} is greatest in the first phosphate group, which is hydrolyzable with myosin. This provides evidence that reactions involving the hydrolysis of adenosine triphosphate to adenosine diphosphate occur somewhat more often than do reactions involving the formation of adenylic acid from adenosine triphosphate, in the intact animal. Such reactions have been demonstrated frequently in studies *in vitro* (15). Whether myosin is the enzyme which controls the turnover of Phosphate 1 in resting muscle is not known and this might depend on the extent of similarity between the processes of maintenance of muscle tone and contraction. Water-soluble enzymes such as that reported by Sakov (16), which catalyzes the transfer of phosphate from adenosine triphosphate to fructose phosphate, might well be involved in the turnover of phosphate in adenosine triphosphate of resting muscle.

The position of myosin in relation to adenosine triphosphate in working muscles is not at all clarified by these studies. Recent studies with other techniques have demonstrated the enzyme-substrate relationship of these two substances, as well as the influence of adenosine triphosphate on the structural properties of myosin. Thus Engelhardt (17) has produced a specific elongation of myosin threads with adenosine triphosphate, a process which resembles relaxation, and Needham and others (18, 19) have found specific effects of adenosine triphosphate on the viscosity and birefringence of myosin. If adenosine triphosphate were the immediate source of energy for contraction of muscle and its hydrolysis with the liberation of inorganic phosphate were controlled by myosin, and if the rate of its resynthesis in working muscles could be measured by the isotope technique, a large increase in uptake of P^{32} by the adenosine triphosphate in continuously working muscle might be expected. Instead, only a small increase was found. If the labile phosphate of adenosine triphosphate is greatly involved during continuous work, the adenosine triphosphate must be resynthesized from the products of its own hydrolysis.

That the isotope technique does not indicate all the phosphate transformations which occur in muscles was shown in earlier studies in which we were able to produce an 80 per cent turnover of phosphocreatine repeatedly without an increase of the uptake of P^{32} as compared with the phosphocreatine of the control resting muscles (20). This limitation of the usefulness of the isotope technique becomes in itself a commentary on the high degree of chemical organization in muscle tissue. That the phosphate

liberated from the organic phosphates during work does not mix with the highly radioactive inorganic phosphate originally present might be due to the fact that the latter is extracellular. Or if the intracellular inorganic phosphate were also highly radioactive, then the lack of mixing must be due to chemical or physical forces existing within the cell. If we assume that the concentration of inorganic phosphate in extracellular fluid is the same as in plasma and that the extracellular space is the same as the chloride space of muscle, then it is apparent that most of the inorganic phosphate of muscle is intracellular. Information on the rate at which labeled inorganic phosphate enters this intracellular space would be essential for an adequate interpretation of our results on working muscles.

Somewhat more than a half of the labile phosphorus of the adenosine triphosphate (22.4 mg.) was hydrolyzed during a 5 minute period of work. Only 10.3 mg. were resynthesized during a subsequent 15 minute rest period, during which time all of the extra inorganic phosphate produced during the work period had disappeared. Further resynthesis of the adenosine triphosphate was therefore slower and was not quite complete at the end of the hour. In contrast to this, of the 40 mg. of phosphorus hydrolyzed from phosphocreatine during a 1 minute period of work, about 80 per cent is resynthesized during a 4 minute rest period.

When a resynthesis of about 35 per cent of the final concentration of adenosine triphosphate was produced three times in 1 hour by alternate periods of 5 minutes of work and 15 minutes of rest, there was an increase in the radioactivity of the first and second phosphate groups. No such increase was found previously when the 80 per cent turnover of phosphocreatine was produced repeatedly. This may be due in part to the greater reorganization of muscle which occurs in the longer rest period necessary for the partial regeneration of adenosine triphosphate.

SUMMARY

In the continuous turnover of the labile phosphate of adenosine triphosphate which occurs in resting muscle, the uptake of P^{32} is greatest in the first phosphate group, which is hydrolyzable with myosin, and least in the third phosphate group, which is attached to ribose. The distribution of P^{32} in the three groups is approximately in the proportion of 100, 75, and 5 the 1st hour after administration of P^{32} .

In the turnover of labile phosphate of adenosine triphosphate which occurs in working muscle, the uptake of P^{32} is increased only a small amount above that of the resting muscles, even after 180 contractions a minute for 1 hour.

Studies of the resynthesis of adenosine triphosphate following the hydrolysis of about a half of the labile P (22.4 mg.) in 5 minutes showed a resynthesis of about 10 mg. in 10 minutes, at which time all the excess inor-

ganic phosphate had disappeared. Further resynthesis was slow and was not quite complete in 1 hour.

When a turnover of about 35 per cent of the labile phosphorus of the adenosine triphosphate was produced three times in 1 hour, a greater increase in the uptake of P^{32} was found than after a 1 hour period of continuous work.

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EFFECT OF ACYLATING AGENTS ON THE SULFHYDRYL GROUPS OF CRYSTALLINE EGG ALBUMIN

By HEINZ FRAENKEL-CONRAT

(From the Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture, Albany, California)

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The action of phenyl isocyanate, carbon suboxide, and ketene on various proteins has been studied by a number of investigators (1-8). In all of this work attention was focused on the reaction of the amino and phenolic hydroxyl groups of the protein under investigation, although the possibility of interaction of other groups with phenyl isocyanate and carbon suboxide has been recognized (2, 5).

In the course of experiments designed to reduce the hydrophilic properties of egg albumin by blocking of its polar groups, the effect of these three reagents on this protein was investigated. It was observed that, between pH 5 and 6, phenyl isocyanate and carbon suboxide react more readily with the thiol groups than with either phenolic or amino groups. Ketene, on the other hand, reacted with a greater proportion of the amino groups than of the thiol groups of the native protein. The thio esters formed by these reagents were found to resemble similar phenolic esters in their instability toward alkali.

These findings tend to emphasize the need for caution in interpreting the changes produced by reagents with supposedly specific action upon the protein molecule. On the other hand, they suggest the use of reversible thiol acylation as a tool in studies of the rôle of sulfhydryl groups in biocatalytically active proteins.

EXPERIMENTAL

Treatment with phenyl isocyanate was carried out by adding the reagent drop by drop to a well stirred dialyzed solution of repeatedly recrystallized egg albumin (3 to 4 per cent) at room temperature; stirring was continued for 16 to 24 hours. The amount of reagent used was varied from 70 to 130 per cent, by weight, of the amount of protein. The solution was found to change from pH 5.3 to 5.5 during this treatment. Carbon suboxide was prepared according to Hurd and Pilgrim (9). Amounts corresponding to 100 to 200 per cent of the protein were distilled into the protein solutions, containing acetate buffer of pH 5.75, at approximately 10°. Alkali was added as needed to maintain the solution between pH 5 and 6. Ketene

was either passed directly from a generator (10) through similarly buffered egg albumin (at 10° or 30°), or it was collected over petroleum ether, which made it possible to pass measured amounts of the gas slowly through the protein solutions. With the former technique, a rapid stream of ketene was passed through the solution for 15 to 45 minutes with stirring and addition of octanol to prevent excessive foaming; with the latter technique, a 2- to 5-fold amount of ketene (by weight) was used, and it was permitted to act on the protein solution for periods ranging from 4 to 48 hours.

Treatment with any of these reagents led to gradual and partial precipitation of the protein. It was difficult to estimate to what extent this precipitation was due to the formation of less water-soluble protein derivatives or to what extent to surface denaturation in the biphasic system.¹ To establish uniform conditions favorable for colorimetric analysis, all solutions were dialyzed, and insoluble matter removed by centrifugation before any analyses were performed. The amount of soluble protein varied from 50 to 90 per cent, depending upon the type and the intensity of treatment.

Thiol groups were determined after denaturation in 40 per cent urea, according to Mirsky and Anson's modification (11) of the Folin method. An amount of solution containing about 10 mg. of protein was brought to 2.5 ml. by the addition of 1 N sodium chloride;² 8 ml. of urea solution, 1 ml. of buffer, and 0.5 ml. of the phosphotungstic acid solution were then added. The resultant blue color was read after 30 minutes on a photoelectric colorimeter, with a red filter; the proportion of free thiol groups was calculated by comparison of these readings with a standard curve prepared from untreated egg albumin within the range of 5 to 25 mg. If a precipitate formed, it was centrifuged off before the readings were taken.

To determine the lability of the derivative toward alkali, 0.75 ml. of 1 N sodium hydroxide was added to 1 ml. of protein sample, followed by 0.75 ml. of 1 N hydrochloric acid after various time periods. The solutions were then prepared for colorimetric assay by the addition of the above reagents with the exception of the sodium chloride. In a study of the time needed for complete liberation of the thiol groups, the chromogenic value of the protein was found to rise to a maximum, followed by a gradual decrease with prolonged exposure to alkali. The time until the maximum was reached, as well as the subsequent rate of decrease, varied from one protein derivative to another. It appeared probable that these decreases might be caused by secondary reactions; *e.g.*, oxidation of the liberated

¹ In view of this fact it is not possible to correlate the present findings with those of other authors who studied the accessibility or reactivity of the thiol groups of the native protein with various water-soluble reagents.

² The final color was independent of the concentration of sodium chloride within the range used.

thiol groups. This secondary reaction appeared to be the limiting factor in the recovery of thiols from thioacyl groups. This was borne out by the fact that recovery was least complete with the slowly hydrolyzing malonyl derivatives. When 0.1 *N* instead of 1 *N* alkali was used, the rate of thiol liberation was greatly reduced, while that of the thiol-decreasing reaction was not markedly affected; maximal recovery was then far from complete. In control experiments in which untreated egg albumin was exposed to 1 *N* alkali only the secondary oxidative reaction, *i.e.* a slow decrease in the thiol content, was observed.³

Phenolic hydroxyl (plus tryptophane) was estimated by the Herriott modification (6) of Folin's procedure, by both the pH 8 and pH 11 method. Irregularities in the phenol values of treated preparations might have been avoided if the pitfalls and improvements of the method, discussed by Miller (12), had been known at the time these experiments were performed. Protein amino nitrogen was determined by the Van Slyke manometric method (13), a 15 minute reaction period in a blacked out chamber being used (14). With malonyl proteins the precautions mentioned by Ross and Christensen (4) were observed. It is well recognized that none of the methods used can be regarded as strictly quantitative, but they appear well suited for comparative studies, in particular when the observed reaction is reversible, as is the case with acylated phenolic and sulfhydryl groups.

Results

The results of a few representative experiments are summarized in Table I. Since the reagents are gradually decomposed by the aqueous solvent, the extent of acylation must be regarded as indicative of the rate of reaction of various protein groups. As stated, only the fraction of the protein which remains soluble in distilled water could be analyzed colorimetrically. It may be assumed that this is the least modified protein fraction, for independent experiments, to be reported elsewhere,⁴ have shown that extensive acylation, in particular with phenyl isocyanate, tends to decrease the water solubility or absorption of proteins. It thus appeared that phenyl isocyanate or carbon suboxide reacted at pH 5 to 6 more rapidly with the thiol groups than with the amino or phenolic groups of native egg albumin.⁵ The thiophenylurethane bonds formed from phenyl isocyanate were completely hydrolyzed by sodium hydroxide within 2

³ Upon exposure to alkali, β -lactoglobulin and crude papain showed increasing amounts of thiol groups.

⁴ Fraenkel-Conrat, H., and Olcott, H. S., in preparation for press.

⁵ At pH 8 to 9, phenyl isocyanate reacts at an appreciable rate also with the amino groups.

minutes, while about 1 hour was needed for maximal hydrolysis of the thio-malonyl bonds resulting from the reaction with carbon suboxide. Ketene treatment caused acetylation of a considerable proportion of the amino groups, accompanied by some sulphydryl acetylation. The thioacetate groups were hydrolyzed as rapidly as the thiophenylurethane groups. No indication of acylation of the tyrosine was obtained in any of these experiments.⁶

For purpose of comparison, cysteine and glutathione were also treated with carbon suboxide and ketene.⁷ As with the protein, the gaseous reagents had to be used in great excess to achieve acylation of more than half of the thiol groups present. Reduced glutathione was found to react somewhat more readily than cysteine. The comparative rates of hydroly-

TABLE I

*Effect of Acylating Agents on Protein Groups and Stability of Thioacyl Bond Toward Mild Alkaline Hydrolysis**

Reagent	Thiol groups			Amino groups	Phenol and indole groups
	Before hydrolysis	After hydrolysis	Time for maximal hydrolysis		
	<i>per cent</i>	<i>per cent</i>	<i>min.</i>	<i>per cent</i>	<i>per cent</i>
Phenyl isocyanate	42	100	2-10	86	90
	45	100		98	115
Carbon suboxide	38	88	60	108	98
	65	90			100
Ketene	76	93	2	42	107
	80	98		22	

* Expressed as the percentage of the groups present in the original protein.

sis of the amino acid thio esters were similar to those of the corresponding protein ester groups. Complete hydrolysis of the thioacetyl bonds of both was achieved in about 1 minute, while 10 and 60 minutes were needed to break the thiomalonyl bonds and release the glutathione and cysteine, respectively. Again, secondary reactions appeared to interfere with com-

⁶ Although the phenolic groups of *native* egg albumin did not react with carbon suboxide, they reacted readily after the protein had been *denatured* by addition of an equal amount of the detergent, sodium alkyl benzene sulfonate (Nacconol NRSF) (15). The malonyltyrosine groups thus formed could be hydrolyzed at pH 11, in confirmation of the finding of Tracy and Ross (5) who used *native* serum albumin. The thiol groups of egg albumin are rendered autoxidizable by this denaturing agent.

⁷ The reaction of the thiol groups of cysteine and thiol acids with phenyl isocyanate at pH 8 has been described by Gaunt and Wormall (2). The resultant thiophenylurethanes were characterized as alkali-labile.

plete recovery of the thiol groups from the slowly hydrolyzing malonyl-cysteine, while good recoveries were obtained in the other three cases.⁸

The author is indebted to H. S. Olcott for helpful suggestions and criticism, to Miss M. Cooper who performed the amino nitrogen analyses, and to F. E. Lindquist and R. A. O'Connell for the preparation of crystalline egg albumin.

SUMMARY

At pH 5 to 6, phenyl isocyanate and carbon suboxide were found to react with the thiol groups in preference to the amino or phenolic groups of crystalline egg albumin. Ketene treatment caused more rapid acetylation of the amino groups than of the thiol groups. The thio esters formed by any of these reagents were hydrolyzed by alkali at room temperature, permitting almost complete recovery of the protein thiol groups. Such reversible acylation of sulfhydryl groups was also demonstrated in model experiments with cysteine and glutathione.

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⁸ In the case of the carbon suboxide-treated glutathione solution, anomalous results were obtained after exposure to alkali, the intensity of the thiol color test increasing steadily with time.

EFFECT OF FASTING ON THE BLOOD LIPIDS OF MICE

By P. L. MacLACHLAN

(From the Department of Biochemistry, School of Medicine, West Virginia University, Morgantown)

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Recognition of the dynamic nature of depot fat calls attention to the question of the selective utilization of fatty acids in the animal organism. Longenecker (1) observed only slight changes in the proportion of component fatty acids of depot fat during fasting and concluded there was no evidence of selective utilization of unsaturated fatty acids. Quagliariello and Scoz (2)¹ and Hodge and his collaborators (4) made the interesting observation that the degree of unsaturation of adipose tissue fat is increased as a result of fasting. Quagliariello and Scoz suggested that in fat mobilization fat is first hydrolyzed in the fat cell and the free fatty acids then desaturated before entering the blood stream to be transported and catabolized elsewhere. This theory is based on the finding in fat tissue of a true lipase that splits neutral fat, and a dehydrogenase that desaturates fatty acids but not neutral fat. Hodge and his coworkers claim that the changes observed may well arise from a non-specific burning of fat with an iodine number of approximately 80.

The present investigation, designed to determine the changes in the amount and degree of unsaturation of the blood lipids during fasting, appears to support the conclusion that there is a non-selective utilization of stored lipids.

Methods

Male, 3 month-old, albino mice, previously maintained on a diet of either oats and Purina dog chow, or Purina rabbit chow and Purina dog chow, were used. After the initial weighing, the mice were fasted, according to the procedure previously reported (4), for 1, 2, 3, and 4 day periods. Blood samples were obtained from the axillary artery according to the method described by Kuhn (5). The whole blood samples were paired for analysis and analyzed by standard methods for phospholipid (6) and acetone-soluble lipid (neutral fat and cholesterol) (7), from which the total lipid values were calculated. The iodine number of the lipid fractions was determined by the method of Yasuda (8). Preliminary experiments with this method showed that unreliable iodine numbers were obtained for the phospholipid fraction when chloroform was used as the solvent. However,

¹ Cited by Rony ((3) p. 42).

it was found that reliable and reproducible iodine numbers were obtained when a 1:1 mixture of chloroform and ether was used to dissolve the phospholipid. The details of these modifications are being published separately.

DISCUSSION

Data on the blood lipids of fasted mice, previously maintained on a diet of oats and Purina dog chow, with chloroform as the solvent for the phospholipid, are given in Table I. A statistically² significant increase was observed in the acetone-soluble lipid and total lipid of the blood of all the fasted mice, and in the phospholipid after the 1st day of fasting. These results are in agreement with those previously reported (9).

The iodine numbers obtained for the acetone-soluble fraction approxi-

TABLE I

Average Data on Blood Lipids of Fasted Mice Previously Maintained on Diet of Oats and Purina Dog Chow (Chloroform Used to Dissolve Phospholipid)

The $\pm$ values for the lipids represent the standard deviations.

No. of mice	Days fasted	Initial body weight	Loss in body weight	Phospholipid	Acetone-soluble lipid	I No. of acetone-soluble lipid	Total lipid
		gm.	per cent	mg. per cent	mg. per cent		mg. per cent
10	0	21 $\pm$ 1		275 $\pm$ 16.8	277 $\pm$ 21.1	91 $\pm$ 3.5	552 $\pm$ 24.4
10	1	22 $\pm$ 1	12 $\pm$ 3	310 $\pm$ 32.6 (+13)*	339 $\pm$ 47.6 (+22)	95 $\pm$ 4.4 (+4)	649 $\pm$ 77.5 (+18)
12	2	22 $\pm$ 1	21 $\pm$ 3	324 $\pm$ 3.6 (+18)	324 $\pm$ 16.8 (+17)	91 $\pm$ 5.2 (0)	648 $\pm$ 16.4 (+17)
6	3	24 $\pm$ 1	23 $\pm$ 2	359 $\pm$ 25.0 (+31)	346 $\pm$ 16.1 (+25)	89 $\pm$ 4.1 (-2)	706 $\pm$ 20.4 (+28)
6	4	24 $\pm$ 1	27 $\pm$ 2	351 $\pm$ 5.2 (+28)	341 $\pm$ 10.2 (+23)	93 $\pm$ 3.2 (+2)	692 $\pm$ 14.4 (+25)

* The figures in parentheses represent the percentage changes from normal.

mated 90, and showed no statistically significant change from normal as a result of fasting.

Because unsatisfactory results were obtained for the iodine number of the blood phospholipid when chloroform was used as the solvent, experiments were carried out with a 1:1 mixture of chloroform and ether to dissolve the phospholipid. Data on the blood lipids of fasted mice previously maintained on a diet of Purina rabbit chow and Purina dog chow, with chloroform-ether mixture as the solvent for the phospholipid, are given in Table II. In contrast to the increase observed in the phospholipid fraction (Table I) the phospholipid values (Table II) showed no statistically significant change from normal as a result of fasting. However, the maximum

² Fisher's test was used.

fasting value for the blood phospholipid (352 mg. per cent) approximates the maximum blood phospholipid values (359 and 349 mg. per cent) reported in Table I and in a previous publication (9) respectively. This absence of any significant increase in the phospholipid fraction (Table II) results because the level of blood phospholipid in the control animals (328 mg. per cent) is appreciably higher than the control values (275 and 256 mg. per cent) reported in Table I and by the author (9) respectively. The higher level of blood phospholipid in the control animals (Table II) may be due to a change in the stock diet; namely, the substitution of Purina

TABLE II

Average Data on Blood Lipids of Fasted Mice Previously Maintained on Diet of Purina Rabbit Chow and Purina Dog Chow (1:1 Chloroform-Ether Mixture Used to Dissolve Phospholipid)

The $\pm$ values for the lipids represent the standard deviations.

No. of mice	Days fasted	Initial body weight	Loss in body weight	Phospho-lipid	I No. of phospho-lipid	Acetone-soluble lipid	I No. of acetone-soluble lipid	Total lipid
		gm.	per cent	mg. per cent		mg. per cent		mg. per cent
14	0	22 $\pm$ 1		328	83	283	95	611
				± 38.1	± 3.3	± 37.2	± 5.7	± 64.5
12	1	23 $\pm$ 1	12 $\pm$ 2	352	84	360	97	712
				± 20.5	± 2.6	± 57.2	± 3.3	± 59.3
				(+7)*	(+1)	(+27)	(+2)	(+17)
12	2	23 $\pm$ 1	20 $\pm$ 2	338	84	287	92	625
				± 16.7	± 2.2	± 34.7	± 4.6	± 49.1
				(+3)	(+1)	(+1)	(-3)	(+2)
12	3	24 $\pm$ 1	24 $\pm$ 1	335	84	310	94	645
				± 28.6	± 2.2	± 35.6	± 7.3	± 61.3
				(+2)	(+1)	(+10)	(-1)	(+6)
12	4	24 $\pm$ 1	30 $\pm$ 3	323	90	262	95	585
				± 24.4	± 8.9	± 30.8	± 6.8	± 47.5
				(-2)	(+8)	(-7)	(0)	(-4)

* The figures in parentheses represent the percentage changes from normal.

rabbit chow for oats. This resulted in much better consumption of the diet by the mice. These results appear to indicate, therefore, that any changes observed in the blood phospholipid level as a result of fasting depend on the prefasting level.

The acetone-soluble lipid and the total lipid (Table II) showed statistically significant increases (27 and 17 per cent, respectively) only on the 1st day of fasting. The total lipid value of the blood for the control animals (611 mg. per cent) was appreciably higher than the control values (552 and 531 mg. per cent) reported in Table I and by the author (9) respectively. This is largely a reflection of the elevated prefasting blood phospho-

lipid value, and tends to minimize the percentage change in the total lipid of the blood following fasting, in comparison to the acetone-soluble lipid.

These results are, however, in substantial agreement with the statement of Rony ((3) p. 29), that there is regularly a slight increase in the neutral fat content of plasma after a 20 to 24 hour fast, usually not exceeding 20 per cent of the postabsorptive value. But as the fast continues, no consistent changes are found in the total lipid or in any of the lipid constituents. Apparently in fasting the depleted tissues may absorb mobilized fat from the blood at about the same rate as it pours into the blood from the depots.

The iodine numbers of the phospholipid and acetone-soluble lipid (Table II) showed no statistically significant change from normal as a result of fasting. When the phospholipid fatty acids are computed as two-thirds of the phospholipid, the average iodine number for the phospholipid fraction (85) closely approximates the value (124) reported by Boyd (10) for the phospholipid fatty acids of human blood plasma. The degree of unsaturation of the blood lipids (Table II) appears to support the conclusion of Hodge and his coworkers (4), that during fasting there is no preferential or selective utilization of the fatty acids but rather a non-specific burning of fat with an iodine number of approximately 80.

SUMMARY

On fasting, 3 month-old, male, albino mice showed a statistically significant increase in the acetone-soluble lipid and total lipid of the blood. Changes observed in the blood phospholipid level, however, appeared to depend on the prefasting level.

No significant changes from normal were found in the degree of unsaturation of the phospholipid and acetone-soluble lipid fractions. These results support the conclusion that there is no preferential or selective utilization of fat during fasting.

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REDUCING SUBSTANCES IN URINE

IMPROVED PRECIPITATING AGENTS

By MARLOWE DITTEBRANDT, MARY TENNEY, AND EDWARD S. WEST
(From the Department of Biochemistry, University of Oregon Medical School, Portland)

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Laug and Nash (1) have shown that urine filtrates prepared by the acid HgSO_4 - BaCO_3 technique of West and Peterson (2) give values for fermentable sugar which are somewhat too high. This was shown to be due to hydrolysis of some precursor of fermentable sugar by the acid of the reagent. The effect could be obviated or minimized by neutralizing the mixture of reagent and urine quickly after mixing. The writers have confirmed the findings of Laug and Nash and believe they have succeeded in developing two improved precipitation methods which overcome this difficulty. In one $\text{Fe}_2(\text{SO}_4)_3$, Lloyd's reagent, and BaCO_3 are used. The other, in which $\text{Fe}_2(\text{SO}_4)_3$, Lloyd's reagent, and PbCO_3 are employed, has been found far superior to all previous methods in removing the non-fermentable reducing materials of urine. In some cases no non-fermentable reducing materials have been found in filtrates prepared by the latter procedure.

Reagents—

Ferric sulfate. Approximately 20 per cent $\text{Fe}_2(\text{SO}_4)_3$ by volume; prepared by dissolving 30 gm. of Mallinckrodt's analytical reagent $\text{Fe}_2(\text{SO}_4)_3$ + water and diluting to 100 cc.

Barium carbonate. Mallinckrodt's technical, precipitated BaCO_3 .

Lead carbonate. Mallinckrodt's pure.

Lloyd's reagent. Product of Eli Lilly and Company.

$\text{Fe}_2(\text{SO}_4)_3$ -Lloyd's Reagent- BaCO_3 Method—10 cc. of urine are added to 55 cc. of water in a 500 cc. Erlenmeyer flask. 15 cc. of the ferric sulfate reagent are added and then 4.0 gm. of Lloyd's reagent. The mixture is thoroughly shaken and allowed to stand for 3 to 4 minutes. Solid BaCO_3 (35 to 40 gm.) is added in small portions with shaking until most of the CO_2 has been evolved. A rubber stopper is placed in the flask which is shaken vigorously. The stopper is removed to permit the escape of CO_2 . Shaking is repeated until no more pressure develops in the stoppered flask. The mixture should not redden blue litmus. If it does, a small amount of BaCO_3 is added and the shaking repeated. The mixture is filtered on a small Buchner funnel under light suction, with a retentive paper of good grade. The filtrate is made acid to Congo red paper with a drop or two of H_2SO_4 and filtered to remove BaSO_4 . A part of the filtrate is fermented with washed yeast according to Somogyi (3). Samples of fermented and unfer-

mented filtrates are measured into sugar tubes, neutralized to phenol red with drops of 0.5 N NaOH, and reducing values determined with Shaffer-Somogyi Reagent 50 (4) or preferably with Somogyi's micro reagent (5).

Fe₂(SO₄)₃-Lloyd's Reagent-PbCO₃ Method—The procedure is as above up to the point of neutralization, which is accomplished with PbCO₃ (about 40 gm.). After filtration as in the first method, the filtrate is rendered acid to Congo red paper by adding a drop or two of sulfuric acid. The filtrate is treated with H₂S to remove traces of lead and the excess H₂S is blown out with a stream of moist air. The mixture is filtered and fermentable and non-fermentable reducing substances are determined on the filtrate as in the first method.

TABLE I

Reducing Substances in Urine Filtrates Prepared by Iron and Mercury Methods
The results are expressed as mg. of glucose equivalent per 24 hour sample.

Subject	Iron filtrates		Mercury filtrates	
	Non-fermentable	Fermentable	Non-fermentable	Fermentable
L. F. N.....	592	63	694	104
M. E. T.....	247	49	276	50
E. S. W.....	385	139	411	155
"	394	140	333	180
"	360	132	348	158
"	415	87	384	94
"	331	142	376	181
"	381	110	372	108
J. E. S.....	365	92	462	104
Average.....	386	106	406	126

EXPERIMENTAL

Fermentable and Non-Fermentable Reducing Substances in Fe₂(SO₄)₃-Lloyd's Reagent-BaCO₃ and HgSO₄-BaCO₃ Filtrates of Urine—In order to compare the reducing substances of urine as determined on acid HgSO₄-BaCO₃ and Fe₂(SO₄)₃-Lloyd's reagent-BaCO₃ filtrates of urine, simultaneous analyses were made on filtrates of a series of 24 hour urine samples. The results are shown in Table I. The fermentable reducing values on the mercury filtrates agree with those of the iron filtrates in some instances, but average about a third higher, confirming the findings of Laug and Nash that the high acidity of the mercury reagent may hydrolyze some precursor in urine to increase the fermentable reducing material. The pH of the urine solution with the mercury reagent added (before neutralization) is about 0.75 by the glass electrode. The corresponding

value for the iron reagent is around 1.6 pH. Samples of urine were precipitated by the iron method and after being mixed with the reagent were allowed to stand 20 minutes before neutralization. The fermentable reducing values were the same as were found on simultaneous controls neutralized immediately after addition of the iron reagent. It will be observed that the iron precipitation is equal or superior to the mercury method in removing non-fermentable reducing materials from urine. Both methods of precipitation give similar results when applied to pure glucose solutions.

West, Lange, and Peterson (6) and Laug and Nash (1) observed no appreciable effect of hydrolysis upon the non-fermentable reducing fraction of mercury filtrates of urine. Fermentable reducing values were definitely

TABLE II

Effect of Hydrolysis before and after Precipitation upon Reducing Substances of Urine; Fe₂(SO₄)₃-Lloyd's Reagent-BaCO₃ Filtrates

The values are expressed as mg. of glucose equivalent per 24 hour sample.

Subject	Before hydrolysis		Hydrolysis before pptn.		Hydrolysis after pptn.	
	Non-fermentable	Fermentable	Non-fermentable	Fermentable	Non-fermentable	Fermentable
M. D.....	251	49	312	109	333	231
M. E. T.....	255	107	319	271	344	299
L. F. N.....	325	117	405	293	483	448
E. S. W.....	320	131	410	286	453	333
W. F. A.*.....	284	708	348	750	380	852
M. E. T.....	342	112	456	278	511	403
Average.....	296	204	375	331	417	428

* Mild diabetic.

increased. This is in contrast to similar results obtained upon Fe₂(SO₄)₃-Lloyd's reagent-BaCO₃ filtrates in the present work. When the iron filtrates were hydrolyzed with 1 N H₂SO₄ for 3 hours in a boiling water bath, neutralized with BaCO₃ (in the presence of a little ferric sulfate to remove H₂S from the BaCO₃), and analyzed in the usual way, a sharp increase in both non-fermentable and fermentable fractions was observed. It was also found that hydrolysis of the iron filtrates gives higher values for both fractions than are found in iron filtrates prepared from hydrolyzed urine. This suggests that when urine is hydrolyzed before precipitation some of the reducing substances, both fermentable and non-fermentable, are destroyed by reaction with materials which are removed by iron precipitation. These points are illustrated by the data of Table II. A rather unexpected result was obtained upon a series of urine samples

from a diabetic on a controlled diet. The samples gave strong tests for acetone bodies. The patient was receiving a diet of 35 gm. of carbohydrate, 55 gm. of protein, and 220 gm. of fat. The fasting blood sugar was about 190 mg. per cent. The urine samples were hydrolyzed before precipitation with $\text{Fe}_2(\text{SO}_4)_3$ -Lloyd's reagent- BaCO_3 . Table III shows the results obtained on four 24 hour samples.

It will be noted that whereas normal urine samples uniformly showed marked increases in fermentable sugar after hydrolysis (Table II) the urine from the diabetic gave essentially no increase. It is realized that this experiment would have been more significant had the filtrates of the urine samples been hydrolyzed, but this could not be done as the experiment was performed previous to the discovery of the effect of direct hydrolysis of the urine. It is unlikely that compensating errors in all samples gave the uniformity of results obtained. As to whether this phenomenon

TABLE III

Reducing Substances in Urine of Diabetic before and after Hydrolysis

The values are expressed as mg. of glucose equivalent per 24 hour sample.

Sample No.	Before hydrolysis		After hydrolysis	
	Non-fermentable	Fermentable	Non-fermentable	Fermentable
1	262	136	319	141
2	251	123	364	134
3	362	321	487	298
4	304	319	415	334
Average.....	295	225	396	227

is characteristic of the diabetic state or of the particular individual, we cannot state. We believe the results are not related to the restricted dietary of the subject because urine on the 2nd day of fasting from two normal individuals showed marked increases in fermentable sugar after hydrolysis. The non-fermentable reducing fraction of the diabetic urine increased after hydrolysis, as does that of normal urine. The results seem to show that there may be precursors in diabetic urine which yield non-fermentable reducing substances upon hydrolysis without giving appreciable fermentable material, or, if the fermentable and non-fermentable fractions arise from different precursors, the urine of the diabetic contained only the non-fermentable precursor. The latter is possibly more reasonable.

While the fermentable and non-fermentable values on $\text{Fe}_2(\text{SO}_4)_3$ -Lloyd's reagent- BaCO_3 filtrates of urine are similar to those on acid HgSO_4 - BaCO_3

filtrates, the precipitating agents must remove different fractions. The mercury precipitation removes material from urine which appears in iron filtrates and causes increased non-fermentable reducing values after hydrolysis.

Comparison of Iron-Lloyd's Reagent-BaCO₃ and Iron-Lloyd's Reagent-PbCO₃ Precipitation Methods As Applied to Urine and Blood—Of many precipitation combinations tried by the writers, that of Fe₂(SO₄)₃, Lloyd's reagent, and PbCO₃ has proved most efficient in removing non-fermentable reducing materials from urine. With it all of these substances have been removed from a number of urines and the amount greatly diminished

TABLE IV

Reducing Substances in Fe₂(SO₄)₃-Lloyd's Reagent-BaCO₃ and Fe₂(SO₄)₃-Lloyd's Reagent-PbCO₃ Filtrates of Urine

The values are expressed as mg. of glucose equivalent per 24 hour sample.

Subject	Fe ₂ (SO ₄) ₃ -Lloyd's reagent-BaCO ₃		Fe ₂ (SO ₄) ₃ -Lloyd's reagent-PbCO ₃	
	Fermentable	Non-fermentable	Fermentable	Non-fermentable
H. S.....	118	266	109	0
R. F.....	59	318	57	169
“ diluted 1:1.....	55	220	114	0
K. B.....	0	90	0	20
“ fasting urine.....	13	28	15	0
E. W.....	57	377	67	243
“ diluted 1:1.....	63	379	67	200
“ “ 1:2*†.....	370	740	387	0
M. D.*.....	59	159	59	0
P. H.*.....	93	183	85	68
E. W.; diluted 1:2*.....	143	192	123	0

* Somogyi micro reagent used (5).

† This sample not the same as the two samples from E. W. preceding.

in others. In certain instances complete removal has been attained by sufficient dilution of the urine before precipitation. Table IV gives the reducing substances of 24 hour urine samples as determined on Fe₂(SO₄)₃-Lloyd's reagent-BaCO₃ and Fe₂(SO₄)₃-Lloyd's reagent-PbCO₃ filtrates.

The Fe₂(SO₄)₃-Lloyd's reagent-BaCO₃ precipitation of urine yields filtrates which on the average contain somewhat less non-fermentable reducing material than is found in acid HgSO₄-BaCO₃ filtrates. The data of Table IV show the Fe₂(SO₄)₃-Lloyd's reagent-PbCO₃ procedure to be much more efficient than either of these methods in removing non-fermentable reducing materials. All of these reagents are rather efficient in removing creatinine from dilute solutions such as urine.

Filtrates of blood prepared by the two iron methods give the same sugar values. While the writers have not applied the $\text{Fe}_2(\text{SO}_4)_3$ -Lloyd's reagent- PbCO_3 procedure to tissue precipitation for sugar determination, it should probably be superior to the mercury and other methods in common use.

SUMMARY

Two improved methods of precipitating urine are described, based upon the use of $\text{Fe}_2(\text{SO}_4)_3$ and Lloyd's reagent, followed by neutralization with BaCO_3 or PbCO_3 .

By use of the $\text{Fe}_2(\text{SO}_4)_3$ -Lloyd's reagent- PbCO_3 procedure most of the non-fermentable reducing substances of urine can be removed. In some cases all of these materials are precipitated.

Some further observations upon hydrolyzable constituents of urine have been made.

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THE ESTIMATION OF THE DICARBOXYLIC AMINO ACIDS IN PROTEIN HYDROLYSATES

By R. KEITH CANNAN

(From the Department of Chemistry, New York University College of Medicine, New York, and the Marine Biological Laboratory, Woods Hole)

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In a recent review (1), Vickery has included aspartic acid and glutamic acid in a group of nine amino acids "for which analytical methods of some accuracy exist." In the discussion of the individual members of this group, however, Vickery acknowledges that "the two dicarboxylic amino acids have received far less attention than most of the others," and, in a final appraisal of the trustworthiness of available analytical data on proteins, he is prepared to concede only that, "There are a few values for glutamic and aspartic acid worthy of serious consideration."

All recent estimations of the amounts of these two amino acids yielded by proteins have depended upon a preliminary precipitation of their calcium or barium salts with alcohol according to the original method of Foreman. From this precipitate, the glutamic acid has been separated and weighed as the hydrochloride and the aspartic acid in the form of its copper salt. The difficulties encountered in attaining quantitative results by these procedures are well illustrated by the extensive work of Chibnall and his associates (2, 3). They have shown that the preliminary alcohol precipitation is incomplete and that the precipitate contains important amounts of amino acids other than those sought. Their work illustrates the skilful, tedious, and reiterated alternation of precipitations and fractional crystallizations which are necessary to obtain results that appear to be quantitative. It is evident that a method for the preliminary separation of the acidic amino acids which is, at once, more complete and more specific than is the method of Foreman should simplify the subsequent isolation of pure products.

The present paper is devoted to an outline of procedures which, we believe, achieve this objective. The essential steps are the adsorption of the acids from the hydrolysate of a protein by a basic resin, followed by their elution from the resin with hydrochloric acid. The solution obtained contains no more than traces of amino acids other than the dicarboxylic acids. From it, glutamic acid hydrochloride and copper aspartate may be crystallized directly in pure form and more readily than from Foreman's fraction.

There have recently become available several inexpensive synthetic resins which act as efficient materials for ion exchange. One of these is a

polyamine, formaldehyde resin which is known as amberlite IR-4.¹ The properties of ion exchange of this and of related materials have been studied and reviewed by Myers (4). The resin is an insoluble basic substance which forms insoluble salts with acids. It is the latter which act as anion exchange materials in the usual sense of the term. More versatile are the properties of the free base. When added to an acid solution, it acquires a positive charge by combination with protons and, in consequence, removes an equivalent of anions from solution. If sufficient resin is used, the pH of the solution rises to the neighborhood of 7. The resin may then be washed free from ions not electrostatically retained, and the bound acid may be recovered by extracting the washed resin with excess of some other acid. If the bound anions are those of a weak acid, only a small excess of a stronger acid is required to effect the complete removal of the weaker acid. Finally, the resin may be reconverted to the free base by washing it with excess of dilute ammonia or sodium carbonate and then with water.²

Consider a solution of protein which has been hydrolyzed with excess of hydrochloric acid, diluted to a suitable volume, and then treated with sufficient amberlite IR-4 to raise the pH to 6 or 7. The neutral amino acids will be present almost entirely in the form of dipolar ions with zero net charge, the acidic amino acids in the form of monoanions, and the ammonia and the basic amino acids (except histidine) in the form of monocations. The histidine will be partially cationic and partially dipolar. The chief anions present will, therefore, be those of the hydrochloric acid and of the dicarboxylic amino acids;³ and it is these alone which should be adsorbed by the resin. Let a , b , and c represent the equivalents of acidic amino acids, bases (basic amino acids + ammonia), and chloride respectively in the original hydrolysate. Then, if α is the fraction of the total anions which is bound by the resin, and if α_a and α_c are the fractions of the total dicarboxylic amino acids and of chloride respectively which are bound, electrical neutrality in the solution requires that⁴

$$a + c - b = (a + c)\alpha = \alpha\alpha_a + \alpha\alpha_c$$

¹ We are indebted to the Resinous Products and Chemical Company, Philadelphia, Pennsylvania, for supplies of this material.

² When amberlite IR-4 is washed with dilute sodium hydroxide, it combines with considerable amounts of sodium, presumably on phenolic groups. Very extensive washing with water is required to remove this base.

³ Other anions, such as those of phosphoric, sulfuric, and acetic acid (from acetylhexosamines) and of such organic acids as may have arisen from deamination processes, may also be present in small amounts varying with the composition of the protein.

⁴ It is assumed that all of the bases present are in the form of monocations. In the case of a protein rich in histidine, values of α , calculated from Equation 1, might be significantly in error.

and, therefore,

$$= 1 - \frac{a}{a + c} \quad (1)$$

That is to say, the value of α is uniquely determined by the ratio of bases to acids. The corresponding values of α_a and α_c can be arrived at only by experiment, since they depend on the relative affinities of the dicarboxylate and chloride ions for the resin. The simplest assumption that we can make is that adsorption is determined solely by electrostatic forces; *i.e.*, that $\alpha = \alpha_a = \alpha_c$. In this case, it is evident that a substantially complete removal of the acidic amino acids by a single treatment with resin can be anticipated only if (a) hydrochloric acid is present in very large excess

TABLE I
Calculated Values of α (Equation 1)

		<i>a</i>		<i>b</i>	
		<i>mM per gm. protein</i>		<i>mM per gm. protein</i>	
Egg albumin*		1.72		1.485	
Edestin*		2.34		2.54	
Mixture No.	No. of cycles	<i>c</i>	Resin required	α , egg albumin	α , edestin
		<i>mM per gm. protein</i>	<i>gm.</i>		
I	1	50	50	0.971	0.951
	2	60	60	0.996	0.990
II	1	10	10	0.873	0.794
	2	20	20	0.984	0.958
	3	30	30	0.998	0.990
III	1	11	11	0.987	0.977
	2	21	21	1.000	1.000

* Chibnall, Rees, and Williams (5).

relative to the bases, or if (b) the greater part of the bases is removed from solution prior to treatment with the resin. These alternatives will form the basis of Methods I and III for the analysis of proteins which will be described in detail below. A third choice (Method II) remains. In this, a hydrolysis mixture is employed for which the value of α is not very close to unity. The treatment with resin is repeated a sufficient number of times to effect the final complete removal of the acidic amino acids. This repetition may be performed conveniently by adding hydrochloric acid to the neutral filtrate from the previous treatment with resin and neutralizing again by the addition of fresh resin.

In Table I we have recorded the calculated values of α for three hypothetical hydrolysis mixtures of two representative proteins. Mixture I

corresponds with a typical hydrolysate in which 50 mm of hydrochloric acid were used per gm. of protein. Mixture II simulates the solution which would be obtained were the free hydrochloric acid in Mixture I removed by repeated distillation *in vacuo*. Mixture III is such as would result were 90 per cent of the bases removed from Mixture II by means of phosphotungstic acid. The recorded values of α are the cumulative values for the numbers of adsorption cycles to which they refer. It is assumed that, in all cycles after the first, 10 mm of hydrochloric acid per gm. of protein were used to acidify the neutral solution from the previous cycle. The amount of resin required in a cycle is given by $w(a + c - b)$, where w is the weight of resin which will bind 1 equivalent of acid at pH 6. In our experience, the value of w for the moist resin is of the order of 1000 gm. The weights of resin given in Table I have been calculated on this basis.

If we assume that $\alpha_a = \alpha_c$, then we may conclude from Table I that a substantially quantitative removal of the acidic amino acids from Mixtures I and III should be effected in two adsorption cycles. In the case of Mixture II, three cycles should be employed. As a matter of fact, a number of experiments on suitable mixtures of hydrochloric acid and amino acids indicated that amberlite IR-4 exhibits a small but definite selective adsorption of Cl^- relative to the monoanions of glutamic and aspartic acids. The differences between α and α_a are, however, too small to invalidate the general conclusions drawn above, provided care is taken to add sufficient resin to raise the pH of the solution to a level at which the ionization of the dicarboxylic acids is essentially complete; *i.e.*, above pH 6.

EXPERIMENTAL

Preparation of Resin for Use—The resin, as received from the manufacturers, may be freed from soluble materials and fine particles by repeated decantation with water. It should then be washed successively with liberal amounts of 4 per cent hydrochloric acid, water, 4 per cent sodium carbonate, and water, and this cycle of operations should be repeated several times. We remove the loose water from the final material, which is in its basic form, by pressing out on a Buchner funnel, and we store it in the moist condition. It is well to subject the resin to a further acid-alkali-water treatment immediately before use. The purified resin still liberates gas noticeably on treatment with dilute hydrochloric acid and sheds a small amount of color. The amount of nitrogen and of solids which are dissolved after several hours contact with 0.5 M hydrochloric acid is, however, quite negligible. In one test, we found that when 50 gm. of resin were stirred with 0.5 M hydrochloric acid for 5½ hours about 20 mg. of solids, containing 2 mg. of nitrogen, were dissolved.

Experiments with Solutions of Amino Acids—Solutions of a number of

individual amino acids in hydrochloric acid were stirred for 1 to 3 hours with sufficient resin to give a final pH of 6 to 7. Analyses of the filtrates showed that the acidic amino acids were adsorbed completely, whereas no significant adsorption of the neutral or basic amino acids occurred. The adsorbed aspartic or glutamic acids were completely recovered when the washed resin was extracted with a moderate excess of hydrochloric acid.

A series of mixtures of glutamic or aspartic acid in dilute hydrochloric acid was prepared, and varying amounts of sodium chloride, ammonium chloride, or arginine monochloride were added to simulate the bases present in a protein hydrolysate. These mixtures were neutralized with resin, the amounts of chloride and of acidic amino acids adsorbed were determined by analyses of the filtrates, and the values of α_c and α_a were derived

TABLE II

Experimental Values of α_a in Mixtures of Glutamic Acid, Ammonia, and Hydrochloric Acid after Neutralization with Amberlite IR-4

a = mm of glutamic acid, b = mm of ammonia, and c = mm of hydrochloric acid per 1000 ml.

a	b	c	α_a (experiment)	α (Equation 1)
27.2	5.1	45	0.88	0.93
27.2	5.1	31	0.86	0.91
27.2	10.2	45	0.78	0.86
27.2	10.2	31	0.75	0.82
68	42.7	253	0.80	0.87
68	42.7	148	0.74	0.82
68	42.7	95	0.69	0.74
68	42.7	69	0.64	0.69
68	42.7	43	0.57	0.61

and compared with the calculated values of α . The results of a typical experiment are given in Table II.

Methods Used in Determining Dicarboxylic Amino Acids in Proteins

Method I—The protein was hydrolyzed in 6 M hydrochloric acid (50 mm per gm. of protein) for 24 to 30 hours. The hydrolysate was diluted to about 50 ml., the insoluble humin filtered off and washed, and the filtrate and washings were neutralized by stirring for 1 to 3 hours with the necessary amount of resin. About 50 gm. of moist resin were required per gm. of protein. During the neutralization, more of the soluble humin precipitated. The solution was decanted from the resin and the latter was washed repeatedly by decantation. The solution and washings were filtered through paper to trap the humin. The combined filtrates were

acidified with hydrochloric acid (10 mM per gm. of protein) and neutralized with fresh resin. This cycle was, in general, repeated once more to give three adsorption treatments in all. The hydrolysate became progressively diluted by the accumulation of washings. In some cases, the time allowed for adsorption was increased to compensate for this dilution; in others, the solution was concentrated *in vacuo* between successive cycles.

The batches of resin were extracted repeatedly with 0.25 M hydrochloric acid until the pH of the extract fell below 2. About 10 ml. of acid were required per gm. of resin. The extracts and washings were combined, concentrated to about 20 ml. *in vacuo*, and clarified with a very small amount of norit. This solution of the hydrochlorides of the dicarboxylic amino acids was finally taken to dryness and the residue preserved for analysis.

Method II—The free hydrochloric acid was removed from the hydrolysate by repeated distillation to a syrup. The syrup was dissolved, filtered to remove the acid-insoluble humin, and neutralized with resin. About 10 gm. were usually required per gm. of protein. The filtrate and washings were reacidified and treated as in Method I, a total of three or four adsorptions being conducted. The washed resins were extracted exactly as described in Method I.

Method III—The hydrolysate was diluted suitably (6) and 30 per cent phosphotungstic acid was added in slight excess. The solution was heated to about 90° to effect a partial solution of the phosphotungstates, which were then allowed to crystallize overnight. The precipitate was removed, without washing, suspended in hot water containing 1 per cent phosphotungstic acid and 0.25 M hydrochloric acid, and again allowed to crystallize overnight. The precipitate was removed and washed in the usual manner. These procedures were designed to minimize danger of loss of dicarboxylic amino acids in the precipitate rather than to assure a quantitative precipitation of the bases.

The combined filtrates and washings from the phosphotungstates were concentrated to a small volume *in vacuo*, the excess of phosphotungstic acid was removed by amyl alcohol and ether with the usual precautions, and the free hydrochloric acid was then removed by distillation to dryness *in vacuo*. The residue was dissolved and treated as described in Method II, two or three adsorption cycles being performed.

One advantage of Method III is that all humin is removed by the phosphotungstic acid prior to the treatment with resin, and cleaner extracts are obtained. We find that this advantage can be extended to the other two methods in the following way. A solution of phosphotungstic acid is added, drop by drop, to the hot diluted hydrolysate until the precipitate of humin flocculates cleanly. After cooling, this precipitate is filtered and

washed with water. Very little humin and no significant amount of phosphotungstic acid remain in solution. It has been our experience that less than 0.3 gm. of phosphotungstic acid is required per gm. of protein. Under the conditions described, we believe that there is little danger of loss of the basic amino acids in the precipitate, because the phosphotungstates of these amino acids are more soluble in the hot solution than is that of ammonia.

Results

Large Scale Analysis of Crystalline Egg Albumin by Method III—The analysis was made on 55.43 gm. of dry ash-free albumin, which had been prepared by the denaturation, with alcohol, of a thrice crystallized preparation (7) of the protein. Four successive adsorptions were employed. The acid extracts of the third and fourth batches of resin contained only 10 and 2 mg. of nitrogen respectively. This corresponded with less than 1 per cent of the total dicarboxylic amino acid nitrogen. The first two batches of resin were extracted with hydrochloric acid. The extracts were taken to dryness and dissolved separately in strong hydrochloric acid. Two crops of glutamic acid hydrochloride were removed from each extract at 0°. The filtrates were evaporated to dryness *in vacuo*, dissolved in water, and treated with copper carbonate at the boiling point. After the excess of the reagent was filtered off, two crops of copper aspartate were obtained from each fraction. Copper was removed from the filtrates, which were then combined and the original extracts from the third and fourth batches of resin were added. From these "combined mother liquors" (Table III) two further crops of glutamic acid hydrochloride were obtained, followed by two small crops of copper aspartate. After removal of copper once more, a further yield of 90 mg. of glutamic acid hydrochloride was obtained. The final filtrate was evaporated to dryness, dissolved in water, and analyzed for nitrogen, amino nitrogen, α -carboxyl, and dicarboxylic amino acids (9). The results are summarized in Table III, where the weights of the combined air-dried fractions are given. Estimations of the nitrogen and the acidity of the glutamic acid hydrochloride and of the nitrogen and copper of the copper aspartate ($C_4H_5O_4NCu \cdot 4\frac{1}{2}H_2O$) agreed with the theoretical values. In no case have we found it necessary to recrystallize the products obtained from the resin extracts. In this experiment, however, we combined all the copper aspartate fractions, dissolved them in a minimum amount of hot dilute hydrochloric acid, and then neutralized them with an equivalent of sodium hydroxide. We recovered 98.5 per cent of the original amount of copper aspartate. The crops of glutamic acid hydrochloride were also combined and 95 per cent was recovered on recrystallization. The filtrate was evaporated to

dryness, dissolved in hot water, and boiled with excess of freshly prepared copper hydroxide solution. From the filtered solution there separated, over many days, a copper salt quite different in crystalline form from copper aspartate. The air-dried material gave analytical values for nitrogen, amino nitrogen, copper, and carboxyl which were identical with those of an authentic preparation of copper glutamate, and corresponded with $C_5H_7O_4NCu \cdot 2\frac{1}{2}H_2O$. The total recovery of glutamic acid as the hydro-

TABLE III

Analysis of 55.43 Gm. of Egg Albumin (Dry Weight at 105°) by Method III

Fraction	Glutamic acid hydrochloride			Copper aspartate		
	gm.	mm	mm per gm.	gm.	mm	mm per gm.
Resin I.....	9.054	49.30		8.469	30.72	
“ II.....	0.565	3.08		0.213	0.77	
Combined mother liquors.....	1.132	6.17		0.321	1.17	
Total isolated.....	10.750	58.55	1.041	8.993	32.66	0.589
Estimated in residues.....		3.36	0.061		1.04	0.019
Isolated + residues.....		61.91	1.117		33.70	0.608
Results of Calvery (8).....			0.949			0.456*
“ “ Chibnall <i>et al.</i> (5).....			1.092			0.611

Analysis of Uncrystallized Residues

- | | |
|-----------------------|--|
| (a) Total N = 7.00 mm | (d) Secondary COOH = 4.40 mm |
| (b) Amino N = 4.65 mm | (e) Aspartic acid (c - b) = 1.04 mm = 0.019 mm per gm. protein |
| (c) α-COOH = 5.69 mm | (f) Glutamic acid (d - e) = 3.36 mm = 0.076 mm per gm. protein |

* This figure corresponds with the figure given by Calvery for the aspartic acid content of egg albumin. His calculation was made on the assumption that the copper aspartate which he isolated contained $7H_2O$. In a personal communication to the author, Dr. Calvery states that the salt was dried to constant weight in air. It is generally agreed that, under these conditions, the salt contains $4\frac{1}{2}H_2O$. When Dr. Calvery's results are recalculated on the latter basis, they correspond with 0.536 mm of aspartic acid per gm. of protein.

chloride and as the copper salt was 99.0 per cent of the amount submitted to recrystallization.

In Table III, our results are compared with the widely quoted values of Calvery (8) and with the more recent ones of Chibnall *et al.* (5). The protein analyzed by us was prepared by the same method as that analyzed by Chibnall. It will be seen that our recoveries of the two acidic amino acids as crystalline salts definitely exceed those of Calvery, but fall a little short of Chibnall's values. The latter are claimed to approach very close

to maximal values. In our case the analyses of the uncrystallized residues indicated the presence in them of small amounts of acidic amino acids. Indeed, they are consistent with the conclusion that the only amino compounds present are dicarboxylic amino acids, since the amino nitrogen is almost identical with the "secondary carboxyl." The apparent absence of all but a trace of neutral amino acids is good evidence for the remarkable selectivity of the resin. There is a small discrepancy between the total nitrogen and the amino nitrogen. This may possibly represent nitrogen dissolved from the resin. If, following Van Slyke *et al.* (10), we take the difference between the values of α -carboxyl and of amino nitrogen as a measure of the aspartic acid, then the residues contained 1.04 mm of aspartic

TABLE IV
Dicarboxylic Amino Acids

The results are given in mm per gm. of protein.

		Glutamic acid hydro- chloride	Copper aspartate	Mother liquors	Total
Egg albumin	Method I (12.10 gm.)	0.978	0.562	0.180	1.72
	" II (10.48 ")	0.941	0.550	0.197	1.69
	Calvery (8)	0.950	0.456*		1.49
	Chibnall <i>et al.</i> (5)	1.092	0.611		1.70
Lactoglobulin	Method I (9.575 gm.)	1.232	0.711	0.23	2.17
	" III (13.02 ")	1.290	0.718	0.215	2.22
	Chibnall <i>et al.</i> (5)	1.460	0.743		2.20
Edestin	Method II (6.41 gm.)	1.102	0.892	0.31	2.30
	" III (14.4 ")	1.222	0.885	0.23	2.34
	Jones and Moeller (11)	1.305	0.766		2.07
	Chibnall <i>et al.</i> (5)	1.405	0.90		2.31

* See the foot-note to Table III.

acid and 3.36 mm of glutamic acid. Adding these quantities to the amounts of crystalline material isolated, we obtain totals which confirm the results of Chibnall with notable precision. It must be conceded, however, that this treatment of the analytical results obtained on the uncrystallized residues is subject to criticism to the extent to which non-amino acids, such as acetic acid, might be present in the hydrolysate. These acids would be expected to appear in the residues and would be estimated as "secondary carboxyl" groups.

Small Scale Analyses—We have applied Methods I, II, and III to the analysis of egg albumin, β -lactoglobulin, and edestin, using about 10 to 15 gm. of protein in each case. The preparation of β -lactoglobulin used was part of the batch of protein on which Chibnall's recent analysis was

conducted. The edestin was a crystalline preparation. The results are summarized in Table IV, where the method employed is indicated. It will be seen that our recoveries of glutamic acid hydrochloride and of copper aspartate were materially lower than those obtained by Chibnall, though they are, perhaps, as good as could be expected from the small amounts of protein used. In particular, the recoveries of aspartic acid are rather satisfactory. We could probably have improved the yields, somewhat, by a tedious crystallization of small additional crops from the mother liquors, but preferred to estimate the amount remaining in the latter by the method of Kibrick (9). When these estimates are added to the amounts of the two acids which were actually isolated, the totals obtained agree remarkably closely with the results of Chibnall.

SUMMARY

1. The use of a basic resin, amberlite IR-4, for the separation of the dicarboxylic amino acids from protein hydrolysates has been investigated. Conditions under which a quantitative separation of these acids may be obtained are outlined.

2. Estimations of the glutamic acid and aspartic acid in egg albumin, β -lactoglobulin, and edestin by the adsorption method are reported.

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THE ESTIMATION OF THE DICARBOXYLIC AMINO ACIDS BY TITRATION

By ANDRE C. KIBRICK

(From the Department of Chemistry, New York University College of Medicine, New York, and the Marine Biological Laboratory, Woods Hole)

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In this laboratory, we have, for some time, been interested in comparing the amphoteric properties of soluble proteins with the amounts of acidic and basic amino acids found in hydrolysates of these proteins. Now, the numbers of acidic and basic groups in a native protein can be estimated with fair precision, and without undue labor, on small amounts of protein. Rapid and fairly reliable methods of estimating the basic amino acids in a few gm. of protein are also available. On the other hand, approved methods for the estimation of the acidic amino acids require the sacrifice of large amounts of protein. Even so, they have not led to very consistent results in the hands of different analysts and, as the recent work of Chibnall and his associates (1) has shown, are subject to serious sources of error. Chibnall has now devised modified procedures which are claimed to be capable of giving results which are within 1 to 2 per cent of the true values. He concedes, however, that "the whole procedure, which requires several months to carry through to completion, is admittedly very laborious and demands analytical skill of a high order."

The procedures of Chibnall are based upon the preliminary separation of the dicarboxylic amino acids as calcium salts according to the method of Foreman. In a contemporary paper, Cannan (2) describes some new methods in which the acidic amino acids are separated by adsorption on a basic resin and are then eluted with hydrochloric acid. The fraction obtained is much purer than that of Foreman, and the labor involved in the subsequent isolation of crystalline products is materially reduced. Nevertheless, much of the tedium of a long series of fractional crystallizations remains and a considerable amount of protein is required. There is need for a rapid method of analysis which may be applied to small amounts of protein, and which will be capable of giving results in reasonable agreement with those of large scale analyses. It is the purpose of this paper to describe some simple procedures which we have found satisfactory for this purpose. The estimations may conveniently be made on less than 1 gm. of protein.

Theoretical—In any mixture of natural amino acids the total moles of dicarboxylic amino acids will be equal to the difference between the total

equivalents of carboxyl groups and the equivalents of α -carboxyl groups. The total carboxyl groups should be capable of estimation either by the acetone titration of Linderstrøm-Lang, or by a suitably devised electro-metric titration. For the estimation of the α -carboxyl groups, we cannot make use of the excellent method of Van Slyke (3), which is based upon the reaction of amino acids with ninhydrin, because of the anomalous behavior of aspartic acid with this reagent. If, however, there is no significant amount of lysine in the mixture, the α -carboxyl groups will be equal to the equivalents of nitrogen which titrate in the presence of formaldehyde and may be estimated by this familiar procedure. Alternatively, the nitrous acid method of Van Slyke may be used, provided the prolines, as well as lysine, are absent from the mixture.

Although the ninhydrin reaction may not be used in the estimation of the total dicarboxylic amino acids, the fact that ninhydrin reacts with both carboxyl groups of aspartic acid may, as Van Slyke has suggested, be exploited for the estimation of this amino acid. The relationships which we have indicated above may be summarized as follows: Let g , a , and n represent the moles of glutamic acid, of aspartic acid, and of non-acidic amino acids respectively in the mixture. Then,

- | | |
|-------|---|
| (I) | Total COOH groups = $2g + 2a + n$ |
| (II) | Ninhydrin-reactive groups = $g + 2a + n$ |
| (III) | Formaldehyde-reactive N atoms = $g + a + n$ |

It is evident that the combination of observations (I), (II), and (III) permits the estimation of the individual as well as the total dicarboxylic acids.

The chief sources of error which are likely to be encountered in the scheme which has been outlined are the presence of lysine, ammonia, or non-nitrogenous acids in the material analyzed. The latter might include phosphoric acid and such organic acids as might arise from deamination of amino acids or from the decomposition of carbohydrate during the hydrolysis of the protein. Such acids would raise the value of observation (I) and would, consequently, be estimated as glutamic acid. The bases, on the other hand, would lead to low values for aspartic acid, since they would elevate observation (III). The crude amino acid mixtures on which we have tested the scheme are (a) the barium salts precipitated from a protein hydrolysate by a large excess of alcohol and (b) the amino acids adsorbed by amberlite IR-4 and subsequently eluted by hydrochloric acid as described by Cannan (2). It is to be expected that small amounts of the foreign acids and bases mentioned may find their way into the precipitate of the barium salts. The fraction obtained by ion adsorption, on the other hand, should certainly contain no bases, but will include all simple

acids which are present in the hydrolysate. In the fractions of the simple crystalline proteins which we have analyzed by the latter method, we have found no evidence of the presence of significant amounts of foreign acids, but this source of error should be controlled when the analysis of proteins rich in phosphate or carbohydrate is undertaken. It may be worthy of note that any pyrrolidonecarboxylic acid in the mixture analyzed will be estimated as glutamic acid because it will yield 1 equivalent in observation (I) and none in (II) and (III).

Apart from the specific sources of error which have been mentioned, the weakness of the scheme is that the results emerge as differences between pairs of measurements of the same order of magnitude. They are subject to the magnification of errors inherent in such deductions. In mixtures containing a high proportion of non-acidic amino acids, this source of error is further magnified. On this ground, the fractions obtained by ion adsorption are to be preferred to those prepared by the method of Foreman.

EXPERIMENTAL

For the estimation of the total carboxyl groups, we have preferred an electrometric titration in water to the colorimetric titration in acetone and have found it convenient to combine the former with an electrometric formol titration in a single continuous procedure. A volume of solution containing about 1 mM of acidic amino acids is acidified to a pH close to 2, and sufficient potassium chloride is added to give an ionic strength of about 0.3 when the solution is diluted to 25 ml. This solution is then titrated with 0.25 M sodium hydroxide to pH 7 with serial determinations of pH at the glass electrode. Sufficient neutral formaldehyde¹ solution is added, at this point, to give a concentration of about 8 per cent, and the electrometric titration is continued to pH 9.5. During this stage of the titration, it is sufficient to record the readings of pH which fall between 8.5 and 9.5. A control titration of the same concentration of formaldehyde in 0.3 M potassium chloride is made and applied as a correction to the main titration to yield the equivalents of formaldehyde-reactive nitrogen.

The titration in water from pH 2 to 7 is corrected for free H^+ and is then plotted as Curve A. For this correction, we use the relation, $-\log[H^+] = \text{pH} - 0.12$, which we derived experimentally from observations of the pH of dilute solutions of hydrochloric acid in 0.3 M potassium chloride.

Curve A is a composite of the contribution of the α -carboxyl groups of all the amino acids present and that of the secondary carboxyl groups peculiar to the dicarboxylic acids. The formaldehyde titration is a measure of the number of equivalents of α -carboxyl groups. The contribution

¹ Neutral formaldehyde may conveniently be prepared by treating the commercial solution with the basic form of amberlite IR-4.

of the latter to Curve A may be simulated by the curve (Curve B) for this number of equivalents of an acid of $pK_{2.2}$.² Subtraction of Curve B from Curve A yields a curve (Curve C) which should closely reproduce the theoretical curve for the secondary carboxyl groups. Its span should correspond with the moles of dicarboxylic acids present. This curve should be essentially symmetrical about a mid-point whose value will depend upon the proportions of aspartic acid ($pK_2 = 3.9$) and glutamic acid ($pK_2 = 4.3$) present, but may be expected to be close to 4.1. An estimation is not acceptable unless Curve C satisfies these criteria of sym-

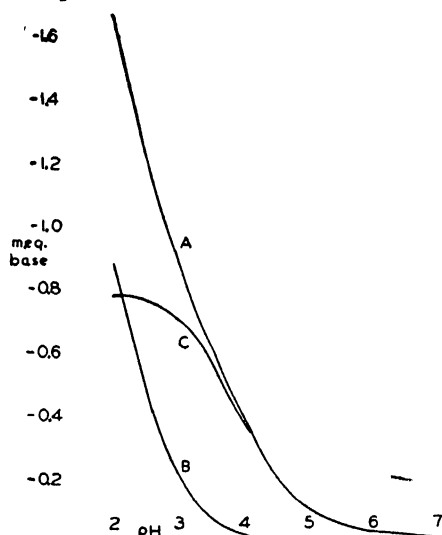


FIG. 1. Experimental titration curves. Curve A, titration in water, corrected for free H^+ ; Curve B, theoretical curve for primary carboxyl groups calculated from formaldehyde titration; Curve C, theoretical curve for secondary carboxyl groups.

metry and position. An example of a set of experimental curves is given in Fig. 1.

The proteins which we have analyzed are listed in Tables I and II. The egg albumin, edestin, and β -lactoglobulin were samples of the actual preparations analyzed by Cannan (2). The duck albumin was a crystalline product prepared by fractional salting-out with sodium sulfate. It differs quite distinctly from hen's egg albumin in amphoteric properties and in electrophoretic mobility. All proteins were hydrolyzed with hydrochloric acid, the excess being subsequently removed by distillation *in vacuo* and the residue was dissolved in water. When these solutions were fractionated by ion adsorption, we followed one or another of the three methods

² This is the mean value, at $\mu = 0.3$, of pK of glutamic acid and aspartic acid. The majority of the amino acids has values not far removed from this.

outlined by Cannan. The hydrochloric acid eluates from the resin were evaporated *in vacuo* to a residue which dissolved in water to give a solution with a pH slightly below 2. Part was reserved for the determination of carboxyl groups by the ninhydrin method and the remainder, after addition of potassium chloride, was titrated as described above.

TABLE I

Analyses of Fractions Separated from Hydrolysates of Proteins by Adsorption on Amberlite IR-4

Method III of Cannan; Three Adsorption Cycles

	M.eq. per gm. protein			mm per gm. protein		
	Total N	Formol N	Ninhydrin-reactive COOH	Dicarboxylic acids = m.eq. secondary COOH	Aspartic acid	Glutamic acid
Egg albumin	1.80	1.77	2.35	1.75	0.58	1.17
	1.80	1.75	2.35	1.70	0.60	1.10
	Isolation methods (4)			1.70	0.61	1.09
β -Lactoglobulin	2.32	2.20	2.95	2.20	0.75	1.45
	2.30	2.24	2.98	2.20	0.76	1.44
	Isolation methods (4)			2.20	0.74	1.46
Edestin	2.35	2.35	3.22	2.26	0.87	1.39
	2.45	2.36	3.17	2.30	0.81	1.49
	Isolation methods (4)			2.31	0.90	1.41

Egg Albumin; Comparison of Three Methods of Cannan

Method No.	mm dicarboxylic acids per gm. protein		
	1 cycle	2 cycles	3 cycles
I	1.55	1.70	
	1.64		
II	1.25	1.53	1.62
	1.34	1.58	1.65
	1.24		
III	1.53	1.75	
	1.64	1.70	
	1.61		
	1.60		

For the preparation of the modified Foreman fraction, excess of barium hydroxide³ was added to a solution of the amino acid hydrochlorides in water. Humins were removed and washed, and the solution was concen-

³ Calcium hydroxide, as used by Foreman, is undoubtedly a more selective precipitant (5), but barium hydroxide probably gives more complete precipitation of the dicarboxylic amino acids. Since our scheme of analysis makes due allowance for impurities, we used barium hydroxide with the object of attaining high yields at the expense of a lower degree of purity.

trated *in vacuo* to about 20 ml. per gm. of protein. 8 volumes of alcohol were added, and the precipitate was removed in the centrifuge after the mixture had remained overnight. The precipitate was washed with alcohol and partially dried *in vacuo* to remove most of the alcohol. It was then dissolved in water, acidified to pH 2 with hydrochloric acid, potassium chloride was added, and the titration was conducted as described.

At the time that the analyses of this modified Foreman fraction were carried out, the improved ninhydrin procedure had not been published. We accordingly sought to supplement observations (I) and (III) by an estimation of glutamic acid from the decrease in amino nitrogen when this

TABLE II

Analyses of Fractions Separated from Hydrolysates of Proteins As Alcohol-Insoluble Barium Salts

	Formol N	Dicarboxylic acids = m.eq. secondary COOH	Glutamic acid (by conversion to pyrrolidonecarboxylic acid)
	<i>m.eq. per gm.</i>	<i>mM per gm. protein</i>	<i>mM per gm. protein</i>
Egg albumin (hen)	2.80	1.71	0.87
“ “ (duck)	2.82	1.71	0.86
	2.74	1.53	0.85
	2.93	1.51	0.81
β -Lactoglobulin	3.17	1.91	1.17
	3.20	1.91	1.22
Edestin	3.44	2.04	1.08
	3.50	2.06	1.16
	3.54	2.08	1.13
Gliadin	4.30	3.34	3.05
	4.22	3.24	3.01
	4.28	3.19	2.87
Isolation methods (5)		3.30	3.19

acid is converted to pyrrolidonecarboxylic acid. Wilson and Cannan (6) have studied the equilibria in this reaction and have suggested that the most favorable conditions for exploiting the reaction analytically would be to adjust the solution of glutamic acid to about pH 4 and to heat at 120° for 6 hours. Under these conditions, they found that the reaction proceeded to an equilibrium corresponding with 92 per cent anhydride formation. Their observations were made in solutions of low ionic strength. In solutions containing 0.3 M potassium chloride, we find an equilibrium of 85 per cent anhydride formation under the above conditions. We have employed the latter figure in the calculation of the results given in Table II.

Results

In Tables I and II are recorded the results of applying the procedures which have been described to the hydrolysates of several proteins. Each

analysis corresponds with a fraction independently separated from the hydrolysate of the protein to which it refers. The results given in the upper part of Table I were obtained by following the scheme outlined in the introduction to this paper. The fractions submitted to the scheme were obtained by adsorption on amberlite IR-4, according to the procedure designated as Method III by Cannan.

The substantial identity of the values for total nitrogen, formol nitrogen, and secondary carboxyl groups in all analyses is convincing evidence that the fractions obtained by adsorption on the resin were relatively pure mixtures of the dicarboxylic amino acids. Moreover, the estimates of the amounts of aspartic acid and glutamic acid in the three proteins examined are in such excellent agreement with the standard analyses of Chibnall as to establish the essential trustworthiness of our scheme of analysis.

In the lower part of Table I, a number of analyses of egg albumin by the three different methods of Cannan are compared. Cannan has considered the numbers of successive adsorption cycles which should theoretically be required to secure a quantitative removal of the acidic amino acids in the three different methods. In our analyses, the numbers of cycles were varied to test the soundness of these deductions in practice. The results confirm the essential validity of the latter. They indicate that two adsorption cycles suffice to give quantitative results by either Method I or Method III. Method II, on the other hand, is much less efficient and we do not recommend its use. There is little to choose in respect to convenience between the two satisfactory methods. In Method I larger amounts of resin are used, which consequently lead to the accumulation of larger volumes of washing fluids and eluates which must subsequently be concentrated by distillation *in vacuo*. In Method III economy in resin is effected by first removing the bases as phosphotungstates. This involves the subsequent removal of excess of phosphotungstic acid from the solution. On the basis of experience, we are inclined to prefer Method III for routine use.

The analyses recorded in Table II were performed on solutions of the crude alcohol-insoluble barium salts. They comprised a titration of the total dicarboxylic acids supplemented by an estimate of the glutamic acid based upon the extent of anhydride formation after 6 hours at 120° and at pH 4. Although the results on each protein are quite reproducible, they are not in consistent agreement with standard analyses.

In the case of gliadin, our analyses may be compared with the results of Chibnall and his associates (5). A more exacting test of the reliability of the procedures used by us is provided by a comparison of our results on the three well defined crystalline proteins. Our recoveries of total dicarboxylic acids agree remarkably well with those of Chibnall *et al.* (4) in the case of egg albumin, but are about 12 per cent low in both edestin and

β -lactoglobulin. The validity of the actual titration procedure has now been established by its successful application to the fractions obtained by the adsorption method. It is probable, therefore, that the irregularity of our results with the modified Foreman procedure indicates that a single precipitation of the barium salts cannot be relied on to effect a complete separation of the dicarboxylic acids.

Our estimates of the amounts of glutamic acid by conversion to pyrrolidonecarboxylic acid are even less satisfactory. In the three crystalline proteins they account for only about 80 per cent of the amounts isolated by Chibnall *et al.* Thus, a considerable discrepancy remains even after allowance is made for a loss of 12 per cent in the precipitation of the barium salts. The explanation for the discrepancy has not been found. Until it shall have been explained or eliminated, we cannot recommend the conversion to the anhydride as a means for the estimation of glutamic acid in mixtures of amino acids. Although we have not applied the ninhydrin reaction to solutions of the crude barium salts, we have no reason to doubt that it would provide a satisfactory estimate of the aspartic acid present and so provide an alternative to the estimation of glutamic acid as the anhydride.

SUMMARY

1. A method is described for the estimation of the dicarboxylic amino acids in small amounts of protein. The method comprises an electrometric titration in water and in formaldehyde solutions supplemented by a gasometric ninhydrin estimation.

2. The results of applying these procedures to fractions separated from protein hydrolysates by adsorption on amberlite IR-4 have been uniformly satisfactory. Less satisfactory results were obtained when the estimations were made on solutions of the crude barium salts precipitated from the hydrolysates by excess of alcohol.

3. The estimation of glutamic acid in amino acid mixture by conversion to pyrrolidonecarboxylic acid has been attempted. The results have been unsatisfactory.

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THE ISOLATION OF GLIOTOXIN AND FUMIGACIN FROM CULTURE FILTRATES OF *ASPERGILLUS FUMIGATUS*

By ARTHUR E. O. MENZEL, O. WINTERSTEINER, AND J. C. HOOGERHEIDE

(From the Division of Organic Chemistry, The Squibb Institute for Medical Research, and the Biological Laboratories of E. R. Squibb and Sons, New Brunswick)

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Some time ago Waksman, Horning, and Spencer (1) reported the isolation of a new antibiotic agent, fumigacin, from culture filtrates of *Aspergillus fumigatus*. The authors recorded the melting point (185–187°) and nitrogen content (3.7 per cent) of their crystalline product, but otherwise confined themselves to a description of its biological properties. In a study aiming at a more complete chemical characterization of this compound we abandoned the charcoal adsorption process of Waksman *et al.* in favor of direct extraction of the acidified filtrate with ether or other immiscible solvents. It soon became apparent that two antibiotically active entities were present in these extracts: (1) a neutral, nitrogen- and sulfur-containing compound melting at 195° which was identified as gliotoxin, previously isolated by Weindling and Emerson (2) from culture filtrates of *Gliocladium fimbriatum*; (2) a weakly acidic substance melting at 220° which is free of nitrogen and sulfur, and unquestionably represents the fumigacin of Waksman in pure form.

Fumigacin is levorotatory ($[\alpha]_D = -132^\circ$). Its ultraviolet spectrum exhibits no maximum but merely strong end-absorption. Marked and so far unexplained inconsistencies in the molecular weight determinations by the Rast procedure and titrations for equivalent weight made it precarious to assign a definite empirical formula to the compound. Nor could this uncertainty be entirely resolved by the preparation of derivatives, although the data given by the latter narrowed the range for the molecular size. Fumigacin readily yields a methyl ester with diazomethane. The analytical figures for this ester, especially its repeatedly determined methoxyl content, indicated the composition $C_{30}H_{40-42}O_7$; consequently fumigacin itself would be $C_{29}H_{38-40}O_7$, which is compatible with its carbon and hydrogen content. However, analyses of a crystalline silver salt were in close agreement with the theoretical values for $C_{33}H_{48}O_8Ag$. Other metal salts could not be obtained in pure condition. Various other derivatives were prepared for the purpose of characterizing the remaining oxygen atoms, but here again the analytical results were too ambiguous to permit a choice between the two formulae now considered most likely on the basis of the evidence given above. We ascribe these difficulties to the fact that the

solubility properties of these derivatives are so similar to those of fumigacin that, at least in some cases, contamination with the latter may have occurred. Much as a repetition of these experiments seems desirable, we are compelled to present our findings as they now stand because with the exhaustion of our supply of fumigacin this study had to be discontinued for the sake of more pressing matters.

Since fumigacin has no phenolic or enolic properties, 2 oxygen atoms must be present in the form of a carboxyl group. Its antibiotic activity is quickly abolished when it is dissolved in caustic alkali. This change is accompanied by a rapid rise of the levorotation and results, under suitable conditions, in the precipitation of a crystalline sodium salt. Back titration showed that a second acidic group, presumably formed by the opening of a lactone ring, had been liberated, but the corresponding free acid could not be obtained in crystalline form. Acetyl determinations on fumigacin, though quantitatively unsatisfactory, gave evidence for the presence of one such group. On the other hand, the presence of a free, readily esterifiable hydroxyl group must be considered doubtful. Attempts to acetylate the compound with acetic anhydride and pyridine under a variety of conditions, or with boiling acetic anhydride alone, led to mixtures of variable composition which resembled the starting material in respect to solubility and melting point range. Treatment with benzoyl chloride or *p*-nitrobenzoyl chloride in pyridine failed to yield crystallizable products. More satisfactory results were obtained with carbonyl reagents. Though the reactions with phenylhydrazine and its nitro derivatives were negative, we could secure an oxime and a semicarbazone, both in crystalline form. The nitrogen content of these derivatives, though it did not correlate too closely with the two formulae under consideration, at least assured the presence of one carbonyl group in the molecule. That this group must be ketonic in nature follows from the positive Zimmermann test and the absence of aldehydic properties.

Fumigacin reacts rapidly with bromine and with permanganate either in sodium carbonate or acetone solution. However, no hydrogen uptake was observed in the presence of a platinum catalyst, and starting material was recovered from the reaction. A crystalline bromine derivative (m.p. 178°) was obtained by reaction with approximately 2 atoms of bromine in chloroform, but the analytical data (C 57.93, H 6.53, Br 16.36) could not be reconciled with either of the two formulae considered above.

After completion of this work a paper by Chain, Florey, Jennings, and Williams (3) came to our attention, in which these authors describe the isolation from culture filtrates of *Aspergillus fumigatus* of an antibiotic substance, designated by them helvolic acid. The properties and com-

position of this compound and of its methyl ester leave little doubt that it is identical with the fumigacin described here. The only serious point of divergence is the specific rotation, which is given as -49.4° , while our value in the same solvent is -132° . Since our figure has been repeatedly checked, we are inclined to ascribe the discrepancy to a factual or clerical error on the part of the British authors. The formula assigned to helvolic acid on the basis of titration and crystallographic x-ray data is $C_{32}H_{44}O_8$, which is in agreement with the analysis of our silver salt. However, it should be noted that their figure for the Rast molecular weight (510) fits much better with the C_{28} formula which is favored by most of our own evidence. No mention is made by the British authors of the occurrence of a sulfur-containing entity.

The antibacterial potency of fumigacin per mg. is considerably less than that of other antibiotic substances such as penicillin, gramicidin, tyrocidin, and gliotoxin, but it is of the same order as that of aspergillic acid. Whereas the latter compound acts on Gram-negative as well as Gram-positive bacteria, fumigacin is effective primarily against Gram-positive bacteria.

The toxicity of pure fumigacin was found to be considerably less than reported by Waksman *et al.* This difference may be due to the presence in their preparation of small amounts of gliotoxin, a substance which is considerably more toxic than fumigacin. To judge from the antibacterial efficacy *in vitro*, subtoxic doses of fumigacin should be capable of inhibiting the growth of sensitive pathogenic bacteria such as *Streptococcus haemolyticus* or *Staphylococcus aureus* also *in vivo*. Since peptones, serum, or whole blood does not interfere seriously with its bacteriostatic action *in vitro*, the prerequisites for employing fumigacin as an effective therapeutic agent exist. Results obtained in protection experiments gave some slight promise in this respect. By using amounts close to the maximal tolerable dose and by proper timing of the treatment, protection was afforded to about 50 per cent of mice infected with *Streptococcus haemolyticus*. All the above observations on the biological properties of fumigacin are substantially in agreement with those of Chain *et al.* on helvolic acid, thus strengthening the chemical evidence for the identity of the two compounds.

Our yields of the pure compounds as well as specific inactivation of the gliotoxin moiety in the culture filtrate by chemical means indicated that this substance, and not fumigacin, accounts for most of the antibiotic activity produced by the mold in Czapek-Dox medium. An inquiry into the composition of the crystals yielded by the method of Waksman therefore seemed in order. Through the kind cooperation of Dr. Waksman we had an opportunity to examine his own crystalline material as well as the

active, crystallizable fraction prepared from charcoal adsorbates which he furnished to us.¹ Pure fumigacin could be readily isolated from these materials, and although we were unable to secure the gliotoxin moiety in crystalline form, the evidence presented in the experimental part leaves little doubt that substantial amounts of the latter compound were likewise present. Apparently the charcoal procedure yields in the active fraction, besides the two active constituents, some contaminating substances, most likely decomposition products of gliotoxin, which impede the crystallization of the latter compound. It should be mentioned that the same difficulty was encountered also in our own procedure of isolation when brown sugar was substituted for glucose in the culture medium, although this measure about doubled the yield of crystalline fumigacin.

Aspergillus fumigatus is by no means unique among molds in its capacity to produce two compounds entirely unrelated chemically, both possessing strong antibiotic activity. In the few instances thoroughly investigated generally one of the antibiotic factors greatly preponderated over the other, depending on the culture medium and the conditions of growth. Thus *Aspergillus flavus* when grown on the surface of a tryptone medium will produce large amounts of aspergillic acid (4), but very little of the penicillin-like substance which it forms in submerged culture (5), while the opposite is true when a modified Czapek-Dox medium with certain adjuvant substances is used for submerged cultivation. With *A. fumigatus*, on the other hand, we have not been able to dissociate to any great extent the metabolic processes leading to the production of gliotoxin, on the one hand, and of fumigacin, on the other, except by the obvious means of greatly reducing the inorganic sulfur content of the medium. Though very little gliotoxin is produced in such a medium, the formation of fumigacin is not measurably impaired. It must therefore be concluded that the two processes represent entirely independent metabolic pathways and are in no stage coupled with each other. On the other hand, if the mold is grown in submerged instead of in surface culture, the proportion in which the two compounds are produced is not appreciably altered, but the yields of both are slightly higher. This is in marked contrast to the behavior of *A. flavus* mentioned above. Some change in the ratio does occur, however, when the glucose in the Czapek-Dox medium used for surface cultivation is replaced by brown sugar. The yield of fumigacin is approximately doubled, while gliotoxin production is decreased; as mentioned before, the neutral fraction was in this case contaminated with by-products which prevented the crystallization of gliotoxin. However, the latter difficulty

¹ We wish to express our sincere thanks to Dr. Waksman for his interest and helpful advice and for the material assistance rendered by furnishing the culture of *Aspergillus fumigatus* and the chemical preparations mentioned.

was not encountered when the mold was grown by submerged cultivation in the brown sugar-containing medium devised for this purpose.

EXPERIMENTAL

Microbiological (J. C. Hoogerheide)

Production and Activity of Mold Filtrates—In the routine production of the mold filtrate for chemical studies, *Aspergillus fumigatus* (Strain W84¹) was grown at 24° in 1 gallon flint bottles each containing 1 liter of ordinary Czapek-Dox medium made up with tap water. Optimal activity was usually reached between the 6th and 8th day of incubation, when the filtrate was harvested and extracted with the shortest possible delay. The pH attained at this point was usually around 6.0.

The filtrate activity was measured by the usual serial dilution technique with *Staphylococcus aureus* as the test organism. As a rule, the limiting dilution just causing inhibition of bacterial growth was 1:500 when the medium was made up with distilled water, 1:250 when tap water was used. A more detailed study of the factors governing the production of the two active agents is in progress. However, a few facts are sufficiently established to warrant mention here. Traces of heavy metals appear to play an important rôle. For instance, addition of 1 mg. of zinc sulfate per liter of medium will entirely suppress the production of antibiotic substances without impairing the growth of the mold. Instead, a dark red pigment, possibly the *p*-benzoquinone derivative fumigatin of Oxford and Raistrick (6), is formed. The lower activity resulting from the use of tap water instead of distilled water in the medium may likewise be ascribed to the presence of small amounts of inhibiting metals. This effect was discovered only after the isolation work had been completed, so that the yields given in the chemical part should not be considered as optimal.

The results described in the following were for the most part obtained in small scale experiments in which tap water was used in the medium. Generally only the total activity of the filtrate was measured, but a few preliminary results obtained later by means of a test which permits differentiation between the gliotoxin and fumigacin moieties are included. The differential method is based on the fact that gliotoxin, which contains a disulfide linkage extremely labile to alkali,² is completely inactivated by heating at 100° for 5 minutes in 1 per cent sodium bicarbonate solution. Fumigacin, though it likewise loses its activity by similar treatment with

¹ We are indebted to Dr. J. D. Dutcher of the Division of Organic Chemistry for advance information concerning the chemical properties and structure of gliotoxin, and for supplying the reference specimen derived from *Gliocladium fimbriatum*. A detailed account of Dr. Dutcher's work on the constitution of this compound will be published soon from Cornell University.

sodium *carbonate*, or in the course of several hours when dissolved in cold caustic alkali, is virtually unaffected by sodium bicarbonate under these conditions. Assays of filtrate samples before and after such treatment in conjunction with the known ratio of the activities of the pure compounds against *Staphylococcus aureus* (fumigacin = 1, gliotoxin = 6) thus give a rough measure of the ratio in which both active components are present in the filtrate.

In unmodified Czapek-Dox medium more than 90 per cent of the total activity was found to be due to gliotoxin. On the weight for weight basis, therefore, both compounds should be present in approximately equal proportions. (The yields by actual isolation did not reflect this ratio; in fact usually about 5 times more crude gliotoxin than fumigacin were obtained. However, not too much significance should be ascribed to this discrepancy, as the loss of total activity in the extraction and isolation procedure was considerable.) Continued incubation beyond 1 week caused a considerable drop in total activity, which was found to be entirely due to a gradual destruction of gliotoxin, the fumigacin content remaining virtually unchanged.

The substitution of brown sugar for glucose in the medium decreases the maximum total activity attainable. This is caused by diminished production of gliotoxin, which is only partly compensated for by increased formation of fumigacin. The latter observation is borne out by the markedly greater yield of this compound from such filtrates by chemical isolation. This effect of brown sugar may be connected with the fact that the pH is considerably higher, about 7.0, at the time when the maximum titer is reached. Variation in the carbohydrate (glucose or brown sugar) concentration in the medium between 0.5 and 9 per cent had no appreciable influence on the final titer. Sucrose is about equivalent to glucose, but substitution of the latter by lactose, starch, glycerol, mannitol, sodium lactate, and sodium acetate abolished the ability of the mold to produce antibiotic activity, and very low titers only were obtained with maltose, inulin, salicin, and calcium gluconate, though the mold grew abundantly on most of these media. Addition of peptone to the regular glucose-containing medium likewise caused a marked drop in activity.

When the mold was deprived of its principal source of sulfur by substituting in the medium magnesium chloride for magnesium sulfate, the production of fumigacin was normal (as also shown by isolation) but, as was to be expected, very little gliotoxin was formed.

Marked antibiotic activity is produced when the mold is grown in submerged culture, either in an Erlenmeyer flask shaken on a machine or in a tank provided with stirring and aeration devices. The medium most suitable for this purpose is composed as follows: KH_2PO_4 0.1 per cent, NaNO_3 0.6 per cent, MgSO_4 0.05 per cent, CaCO_3 1.5 per cent, brown sugar

2 per cent. The optimal titer was reached after 6 days. The proportion of the two active agents was not materially different from that obtained by static culture, though the yield of both was slightly better (isolation).

Antibacterial Properties of Fumigacin—Whereas gliotoxin is active against Gram-negative as well as Gram-positive bacteria, fumigacin acts primarily on the latter.

Table I illustrates the inhibitory effect of fumigacin on the growth of several strains of staphylococci and streptococci. The figures for the 4 to 7 hour period give a measure of the bacteriostatic effect, while those in the

TABLE I
Bacteriostatic Effect of Fumigacin

The minimal concentration of fumigacin in mg. per liter necessary to prevent growth in beef heart-0.25 per cent glucose broth for a specified length of time. The controls showed growth in 4 to 7 hours.

	4-7 hrs.	24 hrs.	> 120 hrs.
<i>Staphylococcus aureus</i> , Strain 873.....	1.5	15	80
“ “ (Heatley strain).....	1.5	14	60
“ <i>albus</i> Strain 61.....	1	6	30
“ “ “ 671.....	1	4	25
“ “ “ 806.....	0.8	6	20
“ <i>citreus</i> , Strain 74.....		4	25
<i>Micrococcus tetragenus</i> , “ 653.....	2.5	60	100
<i>Streptococcus haemolyticus</i> , Strain 660.....	1.5	5	10
“ “ “ C203.....	2	25	40
“ <i>viridans</i> , Strain 355.....	39	312	625
<i>Bacillus anthracis</i> , Strain 4094.....	1.5	6	15
“ <i>subtilis</i>		156	2500
<i>Streptococcus spec.</i> (non-hemolytic).....	7	156	625

last column are the concentrations necessary for permanent inhibition and therefore may be taken as indicating the bactericidal efficacy.

The presence of serum in the test medium causes only slight inhibition of the bacteriostatic action.

Toxicity and Protective Experiments—The L.D. 50 for 20 gm. mice, injected intraperitoneally, was found to be 8 mg., or 400 mg. per kilo. This value is based on results obtained with thirty mice, divided in three equal groups, which received 6, 8, and 10 mg. of fumigacin, respectively, per animal. The solution used for injection was prepared by dissolving 1 gm. of fumigacin in about 5 cc. of 1 per cent sodium carbonate solution and diluting it to 100 cc. with sterile distilled water.

Fumigacin when administered in proper dosage was found to afford some protection to mice injected intraperitoneally with 10 to 100 times the

lethal dose of *Streptococcus haemolyticus* (Strain C203). In these experiments the solution of the compound was injected subcutaneously, in order to avoid direct contact with the infective organisms. For effective treatment relatively large doses have to be employed in the early stages of the infection. Small amounts (0.5 to 2 mg.) given in a single dose merely retard death, while several such doses given twice daily for 2 to 3 days may prevent it. Amounts of 2 to 4 mg. administered once or twice shortly after the infection were found to be most effective, but even with this mode of treatment protection was afforded at best to only 50 per cent of all infected mice.

Chemical Studies (A. E. O. Menzel and O. Wintersteiner)

The culture filtrate was acidified to pH 2 with phosphoric acid and extracted three times with ether, the combined extracts equaling the volume of the filtrate. The ether was evaporated to one-tenth of its volume, shaken repeatedly with saturated sodium bicarbonate solution, which removed a biologically inert, dark red pigment, and then exhaustively extracted with 6 per cent sodium carbonate solution. The ether phase yielded on evaporation gliotoxin (see below). The sodium carbonate solution was acidified and distributed several times with benzene.

Fumigacin—The partly crystalline residue from the benzene (7 to 12 mg. per liter of culture filtrate) yielded on repeated recrystallization from methanol pure fumigacin in the form of filamentous needles (about 3 mg. per liter).

Fumigacin melts with some decomposition at 215–220°, depending on the rate of heating. $[\alpha]_D^{25} = -132^\circ \pm 2^\circ$ (0.41 per cent in chloroform). The ultraviolet absorption curve shows only strong end-absorption below 260 m μ with $E_{1\%}^{1\text{cm.}} = 298$ at 234 m μ .

$\text{C}_{27}\text{H}_{36}\text{O}_8$.	Calculated.	C 69.84, H 7.69
$\text{C}_{27}\text{H}_{36}\text{O}_8$.	"	" 69.55, " 8.06
	Found.	" 69.55, " 7.74
		" 69.65, " 7.90

The equivalent weights obtained by titration with standard sodium hydroxide and phenolphthalein on samples weighing 30 to 60 mg. were 514, 560, and 591. Smaller samples (4 to 10 mg.) gave figures of between 400 and 500.

The figures obtained in two acetyl determinations by the method of Elek and Harte (7) were 7.65 and 9.05 per cent. $\text{C}_{27}\text{H}_{36}\text{O}_8 \cdot \text{CO} \cdot \text{CH}_3$ would require 8.61 per cent.

Fumigacin is practically insoluble in water, sparingly soluble in cold methanol and ethanol, and more readily so in acetone, ethyl acetate, ben-

zene, and ether. It is easily dissolved by chloroform, acetic acid, and dioxane.

Silver Salt—60 mg. of fumigacin dissolved in 5 cc. of alcohol were neutralized with 1.07 cc. of 0.1 N NaOH. To 3 cc. of this solution were added about 10 cc. of 1 per cent aqueous silver nitrate solution. The resulting crystalline precipitate was washed with water and dried *in vacuo* at 100° for 2 hours.

$C_{33}H_{43}O_8Ag$. Calculated. C 57.90, H 6.53, Ag 16.27
Found. " 57.77, " 6.56, " 16.14

The remainder was recrystallized by precipitation from chloroform solution with ether.

Found, C 57.82, H 6.57, Ag 16.40

Qualitative Reactions—The following reactions were negative: ferric chloride, Legal, fuchsin sulfurous acid, Tollens, Molisch, Rosenheim, Hammersten (for cholic acid), Jaffé-Tortelli, digitonin. The Zimmermann reaction with *m*-dinitrobenzene for ketones was strongly positive. In the Chabrol-Charonnet test for bile acid (phosphoric acid and vanillin) a strong red color was obtained. Likewise, the Liebermann-Burchard test gave an intense blood-red color. Fehling's solution was slowly but perceptibly reduced at 100°.

In connection with the latter test, which is also given by gliotoxin, it was thought advisable to ascertain whether our preparations were entirely free from the latter compound. Qualitative reactions for sulfur (sodium melt-nitroprusside, sodium plumbite) and a quantitative nitrogen determination on 14 mg. of fumigacin showed this to be the case. Furthermore, 4 mg. dissolved in 2 cc. of Nessler's reagent gave a clear solution which only on prolonged standing deposited a white precipitate, while as little as 0.02 mg. of gliotoxin caused an immediate turbidity.

Action of Alkali—20.84 mg. of fumigacin were dissolved to 2 cc. with 1 N NaOH, and the change in rotation was followed in a 2 dm. tube.

Time, min.....	5	20	35	60	90	120	200
$[\alpha]_D^{25}$, degrees.....	-27.8	-28.8	-34.6	-44.1	-48.9	-56.6	-69.0

After 4 hours a copious precipitate of well formed plates had settled in the solution. The almost linear rise of the levorotation during the period of observation shows that the reaction had not gone to completion when this point was reached. The crystalline sodium salt thus formed was easily soluble in water, alcohol, and butanol and therefore could not be isolated in pure form. Acidification and extraction with ether yielded a resin which could not be crystallized.

A solution of 27.8 mg. of fumigacin in 2 cc. of 0.1 N NaOH was allowed to

stand for 48 hours at room temperature. Titration showed that 0.1095 milliequivalent of alkali had been neutralized. This corresponds to an equivalent weight of 254 for the reaction product, or to half of the molecular weight of fumigacin on the basis of the $C_{29}H_{38-40}O_7$ formula.

The *methyl ester* was prepared with diazomethane in the usual way. It crystallized from methanol in long, fine needles melting at 260–261°. Its specific rotation $[\alpha]_D^{20}$ was $-150^\circ \pm 2^\circ$ (0.57 per cent in chloroform).

$C_{30}H_{40}O_7$.	Calculated.	C 70.27, H 7.87, OCH_3 6.06, mol. wt. 512
$C_{30}H_{42}O_7$.	"	" 69.99, " 8.23, " 6.03, " " 514
	Found.	" 70.04, " 8.03, " 6.06, " " 479 (Rast)
		" 69.97, " 8.09, " 6.10, " " 501 "
		" 6.37

Oxime—50 mg. of fumigacin were boiled for 3 hours in 3 cc. of a filtered solution containing 140 mg. of hydroxylamine hydrochloride and 200 mg. of potassium acetate in 5 cc. of 90 per cent ethanol. The reaction product, precipitated and washed with water, was repeatedly recrystallized from 70 per cent ethanol. It formed fine needles melting at 204–206°. Found, N 2.15.

The *semicarbazone* was prepared in an analogous fashion. After three recrystallizations from 95 per cent alcohol the melting point remained constant at 225–228°. Found, N 7.22.

Gliotoxin—The ether solution, after extraction of the fumigacin fraction with sodium carbonate solution, was dried and evaporated. The partly crystalline residue (about 60 mg. per liter of culture filtrate) was recrystallized repeatedly from ethanol. The melting point of the pure compound (195° with decomposition, in an open capillary) was not depressed by admixture of an authentic specimen derived from *Gliocladium fimbriatum*.² The specific rotation ($[\alpha]_D^{22} = -245^\circ$ in chloroform) and the ultraviolet absorption spectrum ($\epsilon_{\max}$. at 270 μ , 4500; $\epsilon_{\min}$. at 245 μ , 3500) provided further proof for the identity with the reference preparation (8).

$C_{13}H_{14}O_4N_2S_2$.	Calculated.	C 47.85, H 4.32, N 8.59, S 19.65
	Found.	" 48.68, " 4.24, " 8.40, " 19.36
		" 48.77, " 4.56

The carbon figures obtained in this laboratory on preparations from *Aspergillus fumigatus* as well as from *Gliocladium fimbriatum* were consistently too high and are not in accord with the data of Dutcher (8), which led this author to revise the C_{14} formula of Weindling. The revised (C_{13}) formula is undoubtedly correct, since it has been confirmed by degradation.² The possibility that we were dealing in our isolated product with a C_{14} homologue of gliotoxin appeared remote, since the analytical discrepancy should then have been noticeable also in the hydrogen and sulfur values;

nevertheless it seemed desirable to establish the number of carbon atoms in our preparation by other means. A methoxyl determination demonstrated the absence of an additional methyl group which might have been attached to one of the two hydroxyl groups² in gliotoxin. In order to prove the identity of the underlying heterocyclic ring skeleton in both varieties of gliotoxin, we reduced our compound with hydriodic acid, by which treatment gliotoxin from *Gliocladium* has been shown to yield a sulfur-free compound $C_{13}H_{12}O_2N_2$, melting at 122° .² The reduction product obtained from our material melted at the same temperature and showed no depression when mixed with the reference preparation. Analysis of the derivative confirmed the C_{13} formula.

$C_{13}H_{12}N_2O_2$. Calculated.	C 68.40, H 5.26, N 12.28
Found.	" 68.65, " 5.64, " 12.65

The microanalyses reported in this paper were carried out by Mr. J. F. Alicino.

SUMMARY

Aspergillus fumigatus when grown on the Czapek-Dox medium produces simultaneously two antibioticly active agents, gliotoxin and fumigacin. The former substance accounts for the greater part of the antibiotic activity. The production of the two compounds under various conditions has been studied. It has been demonstrated that the crystalline material formerly described as fumigacin (1) is a mixture of fumigacin and gliotoxin. Pure fumigacin has been prepared and characterized in regard to its chemical and bacteriological properties. It appears to be identical with the helvolic acid recently isolated by Chain *et al.* (3) from the same source.

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THE STIMULATORY EFFECT OF THIAMINE AND CERTAIN OF ITS DERIVATIVES ON THE ASSAY OF VITAMIN B₁ BY YEAST FERMENTATION

By H. F. DEUTSCH

(From the Department of Physiological Chemistry, Medical School, University of Wisconsin, Madison)

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During the course of an investigation on the enzymatic destruction of thiamine, the Atkin, Schultz, and Frey (1) ultramicromethod of thiamine assay by yeast fermentation was employed as a possible means of characterizing certain of the end-products. The choice of this method rests on the claim that aside from thiamine and its pyrimidine component no other related substances stimulate the fermentation of yeast. It was soon observed, however, that thiazole and the pyrimidinesulfonic acid resulting from Na₂SO₃ splitting of thiamine were slightly active and further that the percentage stimulation of the known active compounds varied with the concentration. The detailed study of these effects is reported in this paper.

EXPERIMENTAL

To test the validity of the micro fermentation method various compounds known to stimulate were studied in addition to several chemically related compounds. These were (I) thiamine chloride, 3-[(4-amino-2-methyl-5-pyrimidyl)methyl]-5-(2-hydroxyethyl)-4-methyl thiazolium chloride hydrochloride; (II) cocarboxylase, the pyrophosphate of (I); (III) the pyrimidine portion, 4-amino-2-methyl-5-ethoxymethylpyrimidine; (IV) the thiazole portion, 4-methyl-5-(2-hydroxyethyl)thiazole;¹ (V) the sulfonic acid methylpyrimidine resulting from Na₂SO₃ splitting of thiamine, 4-amino-2-methyl-5-methylsulfonic acid pyrimidine; (VI) oxychlorothiamine, 3-[(4-hydroxy-2-methyl-5-pyrimidyl)methyl]-5-(2-hydroxyethyl)-4-methyl thiazolium chloride hydrochloride; and (VII) a mixture of (IV) and (V) prepared by sulfite splitting of thiamine. In addition, the following compounds were tested for stimulatory activity: adenosine triphosphate, muscle adenylic acid, hexose diphosphate, glycerophosphate, pyruvic acid, uracil, 5-aminouracil, guanine, adenine, xanthine, uric acid, allantoin, creatine, guanidine, and methylguanidine.

The sulfonic acid pyrimidine derivative (V) was prepared by the method of Williams *et al.* (2). Purification was effected by recrystallizing three

¹ We are indebted to Merck and Company for analytically pure samples of the first four compounds.

times from water followed by thorough drying. The oxychlorothiamine (VI) was prepared according to the method of Buchman and Williams (3). It was recrystallized three times from a methyl alcohol-ether mixture, dried *in vacuo*, and tested for unchanged thiamine by the thiochrome test.

Preparation (VII) was obtained by the sulfite splitting of thiamine according to the procedure of Mason and Williams (4) by use of Na₂SO₃ at pH 4.8 to 5.0. The excess sulfite was oxidized to sulfate with H₂O₂ and the solution then neutralized to phenolphthalein. The thiochrome method was used to determine whether any unsplit thiamine remained. This mixture of compounds (IV) and (V) was used as such.

The methods and reagents used by Atkin, Schultz, and Frey (1) in the ultramicrodetermination of thiamine were followed except that 2 instead of 5 mg. of yeast² per flask were used. All stock solutions were diluted to the proper volume immediately before use. Regular Warburg flasks of 13 to 14 ml. volume were used with a final substrate volume of 3 ml. All determinations were made in duplicate except when noted and all compounds tested were added on a molar basis. The flasks were shaken at a rate of 85 oscillations per minute at 30°. Gas production was measured during the 2nd hour of fermentation in two successive 30 minute intervals starting 1 hour after addition of yeast to the substrate. Initial and final manometer readings were made while the flasks were shaking in order to minimize equilibrium difficulties experienced in CO₂ manometry.

Experiments listed in Table I with the same numbers were carried out with the same yeast suspensions.

The observed stimulation of CO₂ production by any given compound is expressed as a Δ value, which represents the microliters of CO₂ produced by 2 mg. of yeast during the 2nd hour of fermentation *in excess* of that produced by the control yeast suspension.

Results

The pyrimidinesulfonic acid, oxychlorothiamine, and the solutions resulting from Na₂SO₃ splitting of thiamine were tested for thiamine by the thiochrome method. The oxychlorothiamine contained less than 0.05 per cent thiamine, whereas the other preparations were negative.

Preliminary experiments with thiamine, pyrimidine, and thiazole at a concentration of 1×10^{-10} mole per flask showed that these compounds stimulated in varying degree (see Experiments 1 and 2, Table I). In addition, concentrations of thiamine and pyrimidine from 7.5×10^{-11} to 1.0×10^{-11} mole were used. These experiments indicated that below 5×10^{-11} mole a straight line relationship exists between thiamine concentration and CO₂ production. The same situation appears to hold for pyrimidine except

² Fleischmann's yeast was used routinely in thiamine assay by fermentation.

TABLE I
Effect of Thiamine and Derivatives

The results are given in microliters of CO₂. 2 mg. of yeast were used in 3 ml. of substrate.

Moles added	Experiment No.	Δ thiamine	Δ pyrimidine	Δ pyrimidine-sulfonic acid	Δ thiazole	Δ Na ₂ SO ₃ split thiamine	Δ pyrimidine + thiazole	Δ cocarboxylase
1×10^{-10}	1	232	191		99	76	199	
	2	181	151		79	70	169	
	3	198	155					
7.5×10^{-11}	4	204	151					
5×10^{-11}	5	166	147					
	6	161	139	9				
	7	152	143	8				
	9*	164	113		33			
	10*	152	130		8			
	11†	165			8			
	11a	157	133					
	12	157				35		
	13	87	70	7			75	
	23	143						162
	24	153						172
2.5×10^{-11}	3	106	109					
	8	104	104	8				
	9*	87	75		11			
	10*	104	96		10			
	11†	115			5			
	11a	111	101					
	12	111				16		
	14	101	105				114	
	23	104						114
	24	107						121
1×10^{-11}	3	60	87					
	9*	32	48		2			
	10*	49	53		8			
	11†	59			11			
	11a	57	53					
	12	64				12		
5×10^{-12}	25	96						65
	9*	18	3		1			
	10*	33	31		3			
	25	59						28

* Single manometers.

† Experiments 11 and 11a were run 3 hours apart with the same yeast.

that it is less stimulatory at the higher concentrations and in decreasing concentrations the stimulation approaches and finally exceeds that of thiamine. These results are shown in Fig. 1. In Fig. 2 the results are

graphically expressed as stimulation per millimicrogram (1×10^{-6} gm.) at the various levels used. It can readily be seen from Fig. 2 that the unit stimulation of thiamine increases slowly at the lower concentrations. The same effect, however, is very marked for pyrimidine. These graphs

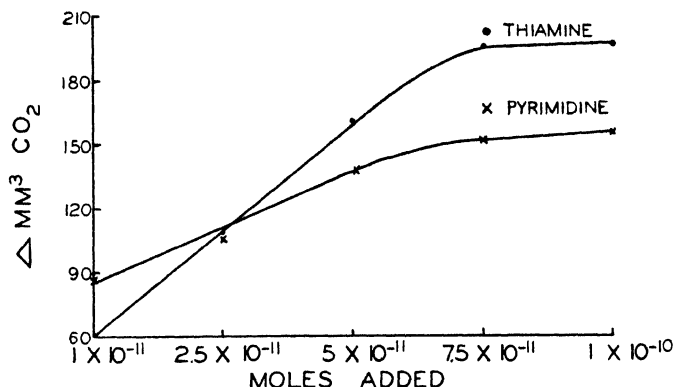


FIG. 1. CO₂ stimulation by thiamine and pyrimidine at different concentrations expressed on a molar basis.

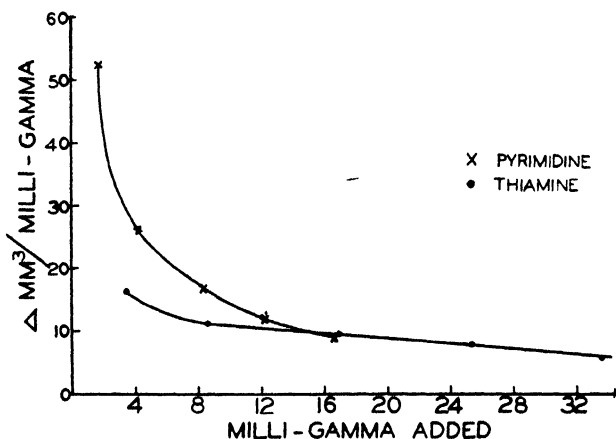


FIG. 2. CO₂ stimulation by thiamine and pyrimidine at different concentrations expressed on a direct weight basis.

strikingly reveal the marked differences in thiamine and pyrimidine stimulation both on a molar and a weight basis.

Further experiments with thiamine, pyrimidine, thiazole, equimolar mixtures of pyrimidine and thiazole, pyrimidinesulfonic acid, and cocarboxylase at various concentrations were carried out. These results are

shown in Table I. Thiazole exhibited a small but definite stimulation at most of the concentrations studied. Mixtures of pyrimidine and thiazole were less stimulatory than the same amount of thiamine at the 1×10^{-10} and 5×10^{-11} mole levels and there is no indication of any appreciable synthesis of cocarboxylase from these compounds under these conditions. The pyrimidinesulfonic acid showed 5.6 to 7.7 per cent of the pyrimidine stimulation at the 5×10^{-11} and 2.5×10^{-11} mole levels. If the stimulation due to a solution of the Na_2SO_3 split-products of thiamine at the 2.5×10^{-11} mole level is compared with the sum of the average values of thiazole and of pyrimidinesulfonic acid at this concentration, it will be seen that they are of similar magnitude. Somewhat the same relation is seen at the 5×10^{-11} mole level. At equimolar levels the stimulatory effect of cocarboxylase was greater than that of thiamine at the higher, but less at the lower concentrations.

Attempts were made to separate thiamine from pyrimidine by use of zeolite filtration. Preliminary experiments revealed that more than 95 per cent of the pyrimidine is also removed by zeolite and that such a separation by this procedure is impossible.

The effect of oxychlorothiamine was compared with that of thiamine at two concentrations, 5×10^{-11} and 2.5×10^{-11} mole. In these experiments CO_2 production was measured not only during the 2nd hour, but also during the 3rd, 4th, and 5th hours after addition of yeast to the substrate. Slight stimulation by the oxychlorothiamine was observed during the 2nd hour, with successive increases thereafter. The values at the two concentrations were practically identical but the percentage of the analogous thiamine stimulation was greater at the lower concentration. These results suggested that the oxychlorothiamine was being slowly aminated to form thiamine. An experiment on a larger scale (150 ml.) with conditions identical to those employed in measuring the CO_2 stimulation by oxychlorothiamine at the 5×10^{-11} mole level was carried out so as to permit chemical assay of thiamine by the thiochrome method. No increase in thiamine was noted in the yeast cells after 5 hours incubation at 30° . Both anaerobic and aerobic systems were also set up containing 1×10^{-6} mole of oxychlorothiamine and 25 mg. of yeast with the same reagents used in the fermentation test in a final volume of 25 ml. These were allowed to incubate for 48 hours at room temperature. There was no increase of thiamine over the controls in the flasks containing the oxychlorothiamine. The fermentation activity of oxychlorothiamine (Table II) thus is not due to synthesis of thiamine from the oxy compound.

The effect of adenosine triphosphate, muscle adenylic acid, glycerophosphate, hexose diphosphate, and pyruvic acid was studied in another series of experiments in concentrations from 1×10^{-6} to 1×10^{-8} mole.

Slight stimulations are shown by all phosphate esters in the concentrations studied, while pyruvic acid was without effect. See Table III.

The following compounds were also tested for stimulatory activity in the fermentation test under the usual conditions, additions being made at the 1×10^{-10} mole level: uracil, 5-aminouracil, guanine, xanthine, uric acid,

TABLE II
Effect of Oxychlorothiamine

The results are given in microliters of CO₂. 2 mg. of yeast were used in 3 ml. of substrate.

Measurement interval	5×10^{-11} mole			2.5×10^{-11} mole		
	Δ thiamine	Δ oxy-chlorothiamine	Per cent of thiamine Δ by oxy-chlorothiamine Δ	Δ thiamine	Δ oxy-chlorothiamine	Per cent of thiamine Δ by oxy-chlorothiamine Δ
2nd hr.....	154	8	5.2	108	8	7.4
3rd ".....	205	17	8.3	130	18	12.8
4th ".....	225	28	12.4	140	27	19.3
5th ".....	257	50	19.4	161	48	29.8

TABLE III
Effect of Phosphate Esters

The results are given in microliters of CO₂. 2 mg. of yeast were used in 3 ml. of substrate.

Substance tested	Δ for 1×10^{-8} mole addition	Δ for 1×10^{-8} mole addition*
Adenosine triphosphate.....	5	
Hexose diphosphate.....	5	18
Glycerophosphate.....	7	19
Muscle adenylic acid.....	7	33

* Average of two experiments.

allantoin, adenine, creatine, guanidine, and methyl guanidine. No stimulation was noted with any of these compounds.

DISCUSSION

From these experiments it is apparent that the interpretation of the effect of thiamine and its breakdown products on the fermentation reaction is in need of revision in several respects.

The effects of pyrimidine, thiazole, and thiamine on the yeast fermentation are different and variations are encountered with these compounds. Both thiamine and pyrimidine have been shown by Schultz, Atkin, and

Frey (5) to be active in the macro fermentation, while thiazole was without effect. In the experiments described pyrimidine was found to be far more active than thiazole in stimulating yeast metabolism when measured in terms of CO_2 production but the latter compound was definitely stimulatory. Ochoa and Peters (6) observed that several pyrimidines containing an amino group in the 4 position greatly stimulated the carboxylase activity of alkaline washed yeast, while thiazole was without effect. In addition they found that thiamine and thiamine monophosphate have a stimulatory effect, although these compounds were apparently not synthesized to cocarboxylase under these conditions. In fact Sperber (7) found that absorption and phosphorylation of thiamine are strongly inhibited by the presence of pyrimidine but not by thiazole. A yeast phosphatase was said to be the active factor in the absorption and phosphorylation of the thiamine. Evidently the pyrimidine portion inhibits the action of this phosphatase. Westenbrink (8) had previously suggested that the stimulatory action of thiamine on cocarboxylase was due to the formation of a thiamine-protein complex which tended to render cocarboxylase more reactive. The same stimulatory effect of thiamine on the carboxylase system of alkaline washed yeast was suggested by Lipton and Elvehjem (9) to be due to the absorption of thiamine by a protein inactive in the carboxylase system, as a consequence of which cocarboxylase is made more available to the active system. Later Westenbrink *et al.* (10) showed that the effect of thiamine was due to the inhibition of a yeast phosphatase, which tends to dephosphorylate cocarboxylase. Nielson (11) has reported that pyrimidine is far more effective than thiazole as a growth promoter for one strain of yeast. He ascribed this to the synthesis of thiazole when pyrimidine is supplied, but it is likely that the effect of pyrimidine alone was due to the enhancement of carboxylase activity, as suggested by the work of Westenbrink. We are aware that alkaline washed dry yeast and living yeast are different entities but the experimental results with the above compounds appear to be similar for these preparations. Various workers have shown that thiazole requirement is greater than pyrimidine for thiamine synthesis by yeasts. Van Lanen *et al.* (12) have found thiazole to be more stimulatory than pyrimidine in aerobic thiamine syntheses and it appeared possible that the yeast may have been able to synthesize some pyrimidine. Fink and Just (13) found pyrimidine alone to be without effect on thiamine synthesis by several yeast species, while thiazole resulted in a slight synthesis. In any event the thiazole and pyrimidine activity in fermentation is reversed in experiments on thiamine synthesis.

From the above considerations it appears that the stimulatory effect of pyrimidine, as well as that of thiamine, in the fermentation assay for vitamin B_1 may be due to their inhibition of a yeast phosphatase which inac-

tivates cocarboxylase. The fact that pyrimidine becomes more stimulatory than thiamine in the lower concentrations may be due to its being more readily diffusible into the yeast cell or its greater affinity for the phosphatase system.

In view of the above reports on thiazole, the slight but constant stimulation encountered with this compound in the assay for thiamine by yeast fermentation does not appear to be due to phosphatase inhibition but possibly to a slight synthesis of thiazole to cocarboxylase. One cannot be sure that synthesis to cocarboxylase is the sole pathway of thiazole activity. The results of the experiments on attempted synthesis involving equimolecular amounts of pyrimidine and thiazole (Table I) do not allow one to draw any conclusions, since pyrimidine activity tends to obscure the picture although the stimulation appears to be additive in the lower concentrations. Chemical assays for thiamine in the systems studied are impractical owing to the extremely small amounts of thiamine that would be formed if such slight synthesis did result.

Our results show that different batches of the same yeast used in these experiments vary as regards the ratio of thiamine and pyrimidine stimulation at a single concentration. This was especially noticeable at 1×10^{-11} mole, at which level the pyrimidine showed from 93 to 150 per cent of the thiamine stimulation. The ratio of thiamine and thiazole stimulation likewise tends to vary both for the same concentration in different runs and for different concentrations during the same run.

The sulfite split-products of thiamine were found to possess activity in this test. When thiamine solutions are split by Na_2SO_3 , thiazole and the sulfonic acid pyrimidine are the end-products. Thiazole, as already mentioned, was found to possess slight activity. The same effect was noted for the pyrimidinesulfonic acid in all experiments involving this substance. Owing to its close structural relation to pyrimidine it is likely that the action of the above sulfonic acid derivative may involve enhancement of cocarboxylase action through the inhibition of the phosphatase which tends to inactivate the latter compound. Schultz *et al.* (14) reported no activity by the pyrimidinesulfonic acid in an unspecified fermentation.

Since Lipschitz *et al.* (15) and Ochoa and Peters (6) have shown that hexose diphosphate catalyzed CO_2 production by alkaline washed yeast, it appeared that certain phosphate esters might catalyze the fermentation. All of the phosphate esters used in these experiments show definite activity at the indicated concentration (see Table III). Owing to the concentrations required for stimulation it is unlikely that the above esters introduce any stimulatory effects in the usual thiamine assay by fermentation. Pyruvic acid was without effect. Uracil, 5-aminouracil, guanine, xanthine, uric acid, allantoin, creatine, guanidine, and methylguanidine showed no

stimulation at the 1×10^{-10} mole level. The average of duplicate determinations on these substances showed ± 2 per cent of the thiamine stimulation at this level and tends to indicate the constancy of fermentation results that were obtained. Schultz, Atkin, and Frey stated that adenylic acid (16) as well as compounds (5) similar or related to 2-methyl-5-hydroxymethyl-6-aminopyrimidine were inactive, although they did not mention the substances tested.

When equimolar mixtures of thiazole and pyrimidinesulfonic acid, prepared by the action of Na_2SO_3 on thiamine solutions, were used in the fermentation assay, different degrees of stimulation were noted, although at no time did the thiochrome test reveal any remaining thiamine. Our results indicated that 22.3 to 14.4 per cent of the thiamine activity remains at the concentrations of 5.0×10^{-11} to 1.0×10^{-11} mole. As previously pointed out, this remaining activity is approximately equal to the sum of pyrimidinesulfonic acid and thiazole stimulation at the 5.0×10^{-11} and 2.5×10^{-11} mole levels. It is evident from Table I that the splitting of thiamine gives rise to products which are not completely inactive in the micro fermentation assay for thiamine.

Variations in the degree of stimulation at equal concentrations were noted in the case of thiamine and cocarboxylase. Peters (17) observed that cocarboxylase was less stimulatory than thiamine in the catatorulin test. This effect was later shown by Banga *et al.* (18) to be the result of cocarboxylase being less diffusible than thiamine. From our data it appears that a like effect may occur with yeast cells at the lower concentrations.

It is thus apparent that these considerations must be taken into account in assaying for thiamine by yeast fermentation. Various investigators (19-21) have used the thiamine and pyrimidine acceleration of yeast fermentation as a means of measuring both of these substances in biological material. They have assumed that Na_2SO_3 treatment completely destroyed the stimulatory effects of thiamine by forming non-stimulatory products. The difference in stimulation between an untreated and a Na_2SO_3 -treated preparation was taken as a measure of the amount of thiamine present. Our results in Table I do not substantiate such a supposition.

Knott *et al.* (19) apparently calculated the stimulation remaining after Na_2SO_3 treatment as micrograms of pyrimidine or that pyrimidine and thiamine were equally stimulatory on a weight basis. Pollack, Ellenberg, and Dolger (22) and Gorham *et al.* (21) have referred to data of Schultz, Atkin, and Frey (5) which they interpreted to indicate that thiamine and pyrimidine stimulate equally on a molar basis.

From Table I of Schultz, Atkins, and Frey (5) it can be seen that on a direct weight basis the pyrimidine is 43.8 per cent more stimulatory than thiamine, while on a molar basis the thiamine is 41.4 per cent more stimula-

tory. Any comparison of activities should be made on a molar basis, since pyrimidine is very likely a metabolic product of thiamine. The expression of the results of thiamine and pyrimidine assay by fermentation by several workers in terms of milliequivalents satisfies this condition (20-22).

In analysis of biological material in which the concentrations of both thiamine and pyrimidine are unknown, any attempt to designate stimulation remaining after sulfite treatment as being due to a definite amount of pyrimidine would seem at best an approximation. Since the difference between the total stimulation and that remaining after sulfite splitting is taken as the thiamine value, this value must of necessity also be approximate in view of the fact that the pyrimidine value cannot be correctly estimated. Schultz, Atkin, and Frey (23) state that in the majority of cases in sulfite treatment of natural materials 90 per cent or more of the fermentation activity is removed and that 99 per cent of the activity of pure thiamine solution can be removed by sulfite. The thiamine method of assay by fermentation has been stated to be more difficult and less certain when the sulfite correction is large (24).

Schultz, Atkin, and Frey in one report (25) state that partial destruction (by splitting or otherwise) of the thiamine molecule causes it to retain its activity in the fermentation test, although they had indicated in another publication (14) that thiazole and the sulfonic acid methylpyrimidine are inactive. We presume that by splitting they refer to the well known action of sulfite on thiamine, although we are aware that hydrolysis of thiamine in solutions of near neutrality and slight alkalinity occurs and gives rise to thiazole and the 5-hydroxymethylpyrimidine derivative. The latter compound, which is presumably the active pyrimidine metabolite occurring in biological fluids, has been stated by the above workers not to be sulfonated by Na₂SO₃ as it occurs in assay solutions. They (25) have suggested the conversion of the thiamine to thiochrome, the latter compound being reported as inactive in the fermentation (26), as a means of estimating the remaining activity due to thiamine breakdown products. Aside from the fact that pyrimidine stimulation varies, the conversion of thiamine to thiochrome estimated by oxidation of a known thiamine solution necessitates the assumption that the percentage oxidation of thiamine in the control and sample is identical. Goodhart and Nitzberg (27) attempted to use the conversion to thiochrome but discarded it because of inaccuracies, resulting largely from incomplete oxidation. However other workers (22, 28) apparently found this method satisfactory in their work. It would appear that the determination of the activity of a thiamine control treated with Na₂SO₃ in each determination would be a logical method of attempting to estimate pyrimidine and thiamine simultaneously. Ochoa and Peters' (6) manometric determination of thiamine through use of alkaline washed dry yeast would appear to be perhaps a more difficult but a better

method, although differences in pyrimidine stimulation at different concentrations would very probably introduce the same difficulty indicated above.

The variation in the response of yeast to thiamine and cocarboxylase is another factor tending to introduce variation. It is obvious from Table I that any attempt at thiamine assay by fermentation in materials also containing cocarboxylase should be preceded by enzymatic conversion of the phosphorylated thiamine to the free form. However, Carleen *et al.* (29) have stated that the method of yeast fermentation is admirably suited to the determination of thiamine in acetate extracts of skeletal muscle.

At this point it might be well to mention that if any data from the experimental results of this paper are to be used for purposes of evaluating the yeast fermentation method for the assay of thiamine, they are applicable only if levels of 5×10^{-11} mole and below are involved. A straight line relationship between thiamine and pyrimidine exists only in this range. Atkin, Schultz, and Frey (1) have clearly indicated that their assay method for thiamine is suitable only under such conditions. Another consideration is the fact that different samples of yeast may vary in their response to a certain level of thiamine but the difference in stimulation between two thiamine concentrations ($\Delta_1 - \Delta_2$) can be used to estimate the amount of thiamine in an unknown sample (5). We experienced one case of extreme variation in thiamine stimulation at the 5×10^{-11} mole level, Experiment 13, showing approximately 50 per cent of the usual stimulation at this level. In all remaining experiments the stimulation appeared to be rather constant at a given concentration, with the exception of Experiment 9 at concentrations below 5×10^{-11} mole.

Our attempts to separate thiamine from pyrimidine by zeolite filtration were not successful. In view of this and the inability to remove thiamine activity completely by treatment with Na_2SO_3 , the present methods of simultaneously estimating both thiamine and pyrimidine in biological material are likely to give results which vary considerably from the true values. This is especially true when the pyrimidine blank is high.

Results obtained with the fermentation assay for thiamine have been shown to be in good agreement with the results of other methods (19, 30, 31). In view of our experimental data it would appear that the active compounds present in biological samples assayed by fermentation cause different and unknown degrees of stimulation. Since the fermentation assay will be the average of these stimulations, the net result will be an intermediate value. Apparently the value so obtained is in agreement with other assays. However, it would appear that in certain instances in which the usual amount of stimulatory substances might vary widely the fermentation assay might give discordant results.

The oxychlorothiamine derivative prepared by deamination of thiamine

has been shown to possess no antipolyneuritic activity in rats (3) and the 4-hydroxypyrimidine analogue does not stimulate carboxylase in alkaline washed yeast (6) or promote the growth of certain microorganisms (32), whereas the 4-amino derivative is active. In these experiments oxychlorothiamine was not converted to thiochrome by alkaline ferricyanide. From our fermentation results (Table II) yeast cells are seen to be activated by oxychlorothiamine as regards CO₂ production. This pronounced enhancement of carboxylase activity with time by oxychlorothiamine suggests that the effect is due to the same type of stimulation of carboxylase activity as that discussed for pyrimidine and thiamine. The effect is not due to synthesis of thiamine and subsequent conversion to cocarboxylase as already indicated. Of interest in this respect is the report of Finkelstein and Elderfield (33) that 1-[(4-amino-2-methyl-5-pyrimidyl)methyl]-2-(2-hydroxyethyl) pyridinium bromide hydrobromide and 1-[(4-amino-2-methyl-5-pyrimidyl)methyl]-2-(2-hydroxyethyl)-3-picolinium bromide hydrobromide show a pronounced ability to stimulate CO₂ production in the yeast fermentation test but possess no antipolyneuritic activity in rats.

SUMMARY

The capacity of thiamine and pyrimidine to stimulate CO₂ production by yeast varies with the concentration of these compounds, thiamine being more stimulatory than pyrimidine at high concentrations, with the reverse obtaining at the lower concentration. Thiamine and cocarboxylase vary in their stimulatory capacity in a similar way. Thiazole and pyrimidine-sulfonic acid resulting from Na₂SO₃ splitting of thiamine also exert a stimulatory effect on the yeast fermentation. Pyrimidine cannot be separated from thiamine by zeolite filtration nor can the stimulatory effect due to thiamine be adequately removed by treatment with Na₂SO₃. Hence the simultaneous estimation of thiamine and pyrimidine as usually performed in the fermentation assay may yield only approximate values. Oxychlorothiamine although it stimulates the yeast fermentation is not converted to thiamine chloride by the yeast used in these experiments. Certain phosphate esters also stimulate the yeast fermentation.

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THE SEPARATION OF CAROTENE FROM VITAMIN A FOR THE DETERMINATION OF VITAMIN A IN BLOOD PLASMA*

By PAUL D. BOYER,† PAUL H. PHILLIPS, AND J. KNOX SMITH

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

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Carotene and other carotenoids interfere in the determination of vitamin A by the Carr-Price reaction. This interference has been amply discussed by With (1). In analysis of certain samples of cattle blood nearly all of the observed color of the Carr-Price reaction may be due to the carotene present. Therefore, in order to determine adequately the vitamin A content of such samples it is necessary to separate the carotene from the vitamin A.

Satisfactory procedures for the separation of carotene from vitamin A are lacking. Phase separation between petroleum ether and methyl alcohol is not satisfactory (2, 3). Chromatographic separation with fibrous alumina (4) or similar substances is not adaptable for routine analytical work, and it is difficult to elute the adsorbed vitamin A quantitatively. Attempts to adapt the norit adsorption method of De (5) to the determination of vitamin A in blood were unsuccessful in our hands. Not only the carotene but also the vitamin A was adsorbed in an appreciable amount by the norit.

A simple method for the separation of carotene from vitamin A has been developed, which is based upon the differential solubilities of carotene and vitamin A in 50 to 60 per cent of ethyl alcohol. The carotene is precipitated from alcohol solutions by dilution, whereas vitamin A remains in solution. The development of the method and its application in analysis of cattle blood are herewith described.

Procedure

The separation is carried out on solutions of absolute ethyl alcohol containing the carotene and vitamin A. Ether or petroleum ether extracts of the material to be studied are made, the solvent evaporated, and the residue taken up in alcohol. Saponification prior to solution in alcohol is desirable

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† Now at the Department of Chemistry, Stanford University, California.

but this step may be dispensed with in certain instances. The directions for the procedure developed follow.

6 ml. of 0.05 per cent sodium chloride in distilled water are added with shaking to 8.0 ml. of an absolute ethyl alcohol solution of the sample in a 50 ml. test-tube. The sodium chloride prevents formation of a colloidal precipitate. The sample is allowed to stand for about 30 minutes in a refrigerator, after which it is filtered on a No. 40 Whatman filter paper. The test-tube and filter are carefully washed with about 10 ml. of a mixture of 4 parts of absolute ethyl alcohol and 3 parts of the sodium chloride solution. The precipitated carotene is removed by this procedure, while the vitamin A and a portion of any xanthophylls present remain in the filtrate.

Absolute methyl alcohol may be substituted for the ethyl alcohol but with less dilution. However, residues from blood extracts are less soluble in methyl alcohol.

The method is adaptable to the removal of carotene from solutions in which the carotene concentration greatly exceeds the vitamin A concentration, but is not suited to the removal of minute traces of carotene from vitamin A solutions.

Preliminary studies in the development of the method indicated that carotene could be separated from vitamin A by precipitation from alcohol solution. Further studies were made to determine the limits within which carotene could be precipitated from alcohol by dilution without loss of vitamin A. For this purpose solutions of crystalline vitamin A alcohol¹ and purified carotene were used. With these solutions it was found necessary to add 0.2 ml. of a saturated solution of lard in absolute ethyl alcohol to increase the bulk of the precipitate and improve the retention of the carotene on the filter. Fig. 1 shows the results obtained when increasing amounts of 0.05 per cent sodium chloride were added to 8.0 ml. of absolute ethyl alcohol containing 4.2 γ of vitamin A and 45 γ of carotene. It is readily seen that the vitamin A remained in solution until the alcohol content was reduced to below 50 per cent, whereas 97 per cent of the carotene was precipitated when the alcohol concentration was reduced to 60 per cent. Thus the desired alcohol concentration for the separation of carotene from vitamin A was between 50 and 60 per cent ethyl alcohol. Although the initial carotene concentration was over 10 times that of the vitamin A in these separation experiments, it was possible to recover 97 to 99 per cent of the vitamin A, while 97 per cent or more of the carotene was removed.

When the separation procedure was applied to a distilled vitamin A concentrate (250,000 I.U. per gm.) or to a shark liver oil (15,000 I.U. per

¹ Obtained from the Eastman Kodak Company.

gm.), it was found necessary to saponify the sample in order to prevent loss of vitamin A (Table I). These results as well as the results obtained with the vitamin A alcohol suggest that the vitamin A must be present in the form of the free alcohol (or possibly as a soluble ester) in order for the separation procedure to be successfully used.

Application in Blood Analysis—The success of the experiments with pure vitamin A and carotene solutions indicated that the method offered good possibilities as an aid to the determination of vitamin A in blood samples containing high concentrations of carotene.

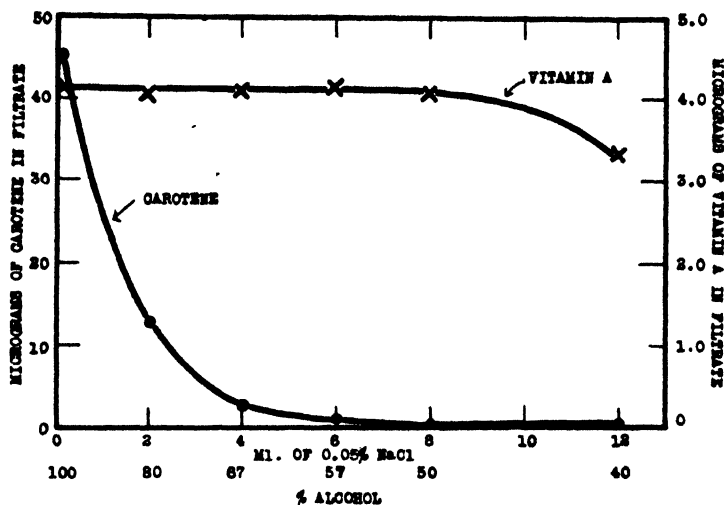


FIG. 1. The effect of alcohol concentration on the vitamin A and carotene in the filtrate from a pure vitamin A alcohol and carotene solution. For the description of the experimental procedure see the text.

In studies on blood samples the vitamin A and carotene were extracted essentially as described by Kimble (6). Yudkin (7) found that this extraction procedure gave good results when compared to ether extraction of previously saponified plasma.

The procedure used for blood analysis was as follows: To 10.0 ml. of plasma in a 50 ml. test-tube fitted with a glass or cork stopper were added 10 ml. of 95 per cent ethyl alcohol and 24.0 ml. of petroleum ether. The stopper was sealed in with mineral oil and the tube and contents shaken gently for 10 minutes. The tubes were then placed in the refrigerator for an hour or more to obtain separation of the layers. A suitable aliquot of the petroleum ether extract (1 to 10 ml.) was diluted to 10.0 ml. with petroleum ether and the carotene estimated as described elsewhere (8, 6, 9).

A 20.0 ml. aliquot, if necessary, including that used for carotene estimation, was next transferred to a 75 ml. Pyrex test-tube. Two such tubes were connected by means of a Y-tube to a water suction pump and the solvent removed under a slowly increased vacuum with rotary shaking of the tubes. When a full vacuum had been applied, the tubes were heated in a water bath at not over 70° until the solvent was barely evaporated. The test-tube and contents were cooled in water, the vacuum released, and the residue dissolved immediately in 8.0 ml. of absolute ethyl alcohol. If saponification was desired, the residue was taken up in alcoholic potassium

TABLE I
Effect of Precipitation and Filtration of Carotene upon Retention of Vitamin A in Various Materials

Material	Vitamin A in alcohol solution			Carotene in alcohol solution			Vitamin A added	Vitamin A recovered	
	Before filtration	In filtrate		Before filtration	In filtrate	Eliminated			
	γ	γ	per cent	γ	γ	per cent	γ	γ	per cent
Pure vitamin A + carotene. . .	4.14	4.01	97	42	1.2	97			
Distilled vitamin A concentrate.	6.24	2.74	44						
Saponified, distilled vitamin A concentrate.	5.40	5.16	96						
Shark liver oil.	4.35	0.34	8						
Saponified shark liver oil. . . .	2.66	2.58	97						
Blood plasma extract, dog. . . .	3.42	1.29	38	None					
Saponified blood, dog.	2.65	2.57	97	"					
" " sheep.	2.38	2.34	98	"					
" " Holstein.	0.98	0.93	95	1.5	0.7	53			
" " Guernsey.		1.74		153	9.3*	94	2.85	2.77	97
" " "		2.04		137	25.2*	82	2.62	2.48	95
Milk extract.	4.40	3.88	88	4.8	0.8	83	3.94	3.35	85

* Mostly xanthophylls.

hydroxide instead of absolute alcohol. If necessary, slight heating facilitated solution of the residue. The carotene was precipitated and removed by filtration as described above. Precipitated material other than carotene gave sufficient bulk to the precipitate to provide an excellent separation. The alcohol content of the filtrate was reduced to about 40 per cent by addition of distilled water and the filtrate was extracted by thorough shaking for 10 minutes with 13.0 ml. of petroleum ether. A good separation of the ether layer was obtained by allowing the mixture to stand in the refrigerator. 10 ml. of this layer were then transferred to an Evelyn colorimeter tube.

In the presence of residual carotenoids the galvanometer deflection with the No. 440 filter was determined. In most cases these were found to be mainly the xanthophylls. The correction factor for the xanthophylls which appear in cattle blood is given in Fig. 2. After evaluation of the xanthophyll correction the petroleum ether was removed under a vacuum as before, the sample dissolved in 1.0 ml. of chloroform, and the vitamin A determined by the Carr-Price reaction (8, 6, 9).

When saponification of the sample was necessary, the above procedure was modified. The residue from the 20.0 ml. of petroleum ether extract was dissolved in about 10 ml. of 20 per cent potassium hydroxide in ethyl alcohol and heated carefully for 15 to 20 minutes in a boiling water bath. An equal volume of water was added, and the contents chilled and then extracted four times with 5 ml. portions of diethyl ether in a separatory

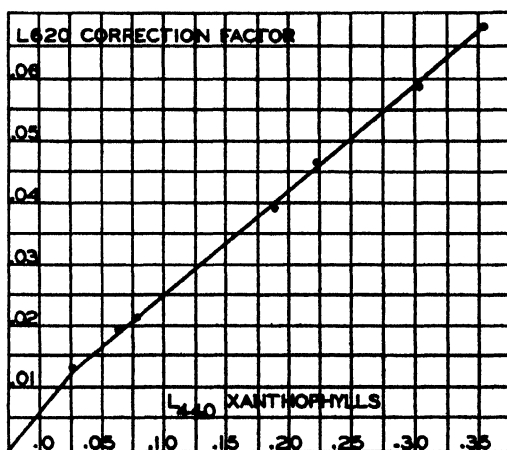


FIG. 2. The relationship between the galvanometer deflection (L_{440}) and the Carr-Price reaction (L_{620}) of xanthophylls from blood plasma of Guernsey cattle.

funnel. The combined ether extracts were washed twice with water, 5 ml. of petroleum ether were added, and the extracts washed twice more. The washings were extracted singly in a separate separatory funnel with another portion of ether which was then added to the original washed extract. The combined extracts were allowed to stand for 20 minutes and any water that settled out was carefully removed. The extract was then transferred to a 75 ml. Pyrex test-tube and the solvent removed under a vacuum as previously described. The residue thus left was ready to be dissolved in absolute ethyl alcohol and the procedure was carried out as outlined for the non-saponified sample.

To check whether loss of vitamin A occurred when the method was applied to blood, samples of the blood plasma from Holstein cattle on limited carotene ingestion and from dogs and sheep were used. These blood plas-

mas had little or no carotene present and thus permitted accurate determination of the vitamin A both before and after application of the separation procedure. The results are shown in Table I. These results show that the method was applicable to blood without loss of vitamin A. Non-saponified extracts gave reliable results with sheep and cattle blood but in the case of the dog blood saponification was necessary to prevent loss of vitamin A which otherwise occurred. These data suggest that the vitamin A in sheep and cattle blood plasma was present as the free alcohol, or as a soluble ester, while in the dog blood plasma it was present as an ester insoluble under these conditions.

In Guernsey cattle the vitamin A content of blood which contained high levels of carotene was equally well determined by this method. Because of the high carotene concentrations, recoveries of added vitamin A alcohol were used as a check on any vitamin A loss. The recoveries were made by comparing the increase in the Carr-Price reaction resulting from additions of vitamin A to duplicate samples before or after filtration. This procedure gave an accurate means of ascertaining whether any of the added vitamin A was retained in the precipitate. Representative results are given in Table I. It is seen that both non-saponified and saponified samples yielded good recoveries of the added vitamin A alcohol when the precipitation-filtration procedure for the separation of carotene was used. However, analyses of non-saponified samples gave values for vitamin A which were only 87 to 95 per cent of those obtained with saponified samples. Thus for the greatest accuracy the sample should be saponified, but for comparative results the non-saponified samples can be used advantageously. Data collected from the unsaponified blood plasma samples of 73 Guernsey cattle gave carotene values ranging from 54 to 2070 γ per 100 ml. of blood plasma, with an average value of 682 γ , while vitamin A values ranged from 3.0 to 29.7 γ per 100 ml. of blood plasma with an average of 16.2 γ per 100 ml.

In further studies on blood plasma in Guernseys the effect of alcohol concentration upon the amounts of carotene, xanthophylls, and vitamin A appearing in the filtrate was determined. Fig. 3 shows the results obtained with a saponified plasma extract from Guernseys. The concentration of the xanthophylls was estimated by phosphoric acid extraction (10). The rapid decrease in the carotene content of the filtrate as contrasted to the slower decrease in xanthophyll content and the maintained vitamin A content is readily evident.

In various trials with Guernsey blood, from 67 to 90 per cent of the carotenoids in the filtrate after carotene precipitation was found to be xanthophylls. Saponification increased the xanthophyll content of the filtrate roughly 3 to 4 times. Hence, a correction for the Carr-Price reac-

tion of the xanthophylls was evaluated. A saponified extract in petroleum ether was chromatographed on calcium carbonate as in the procedure of Wald (11). The position of vitamin A on the column was located by painting with antimony trichloride in chloroform, and the xanthophyll zone carefully separated and extracted with petroleum ether containing a small amount of methyl alcohol. The galvanometer deflection and the Carr-Price reaction of the isolated xanthophylls were then determined. Fig. 2 shows the results of these observations. The relationship between the galvanometer deflection produced by the xanthophylls (L_{440}) and the

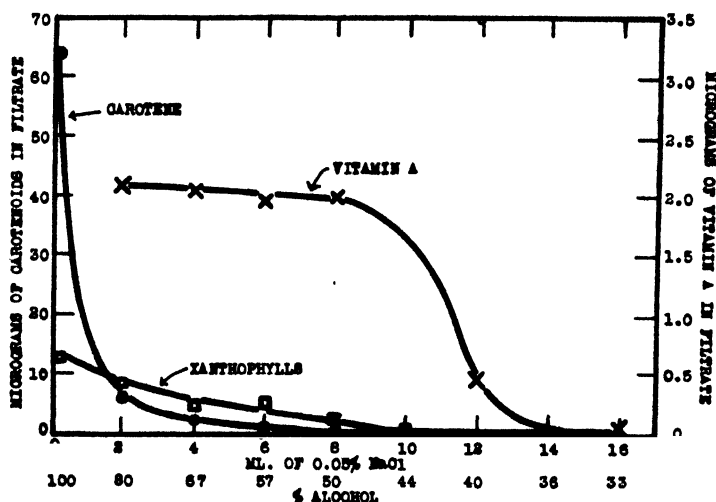


FIG. 3. The effect of alcohol concentration on the carotene, xanthophylls, and vitamin A in the filtrate from a saponified extract of plasma from Guernsey cattle. The xanthophylls are expressed as the micrograms of carotene equivalent to the galvanometer deflection produced by the xanthophylls. For the description of the experimental procedure see the text.

necessary correction to be subtracted from the vitamin A reading (L_{320}) is thus readily discernible.

When applied to milk extracts the carotene precipitation procedure gave fair results, but was not as successful as when applied to blood extracts. Recoveries obtained represented 84 to 93 per cent of the original vitamin A present and 85 to 95 per cent recovery of added vitamin A alcohol. However, milk samples do not normally contain sufficient carotene to warrant its separation from vitamin A.

SUMMARY

A simple procedure for the separation of carotene from vitamin A has been described. The method is based on the differential solubilities of

carotene and vitamin A in ethyl alcohol. The carotene is precipitated from absolute ethyl alcohol solutions by dilution and is separated from the vitamin A by filtration. The method has been successfully applied to solutions of pure vitamin A and carotene and to extracts from various blood samples. It is particularly valuable for the determination of vitamin A in blood samples containing high concentrations of carotene. The method is applicable to solutions containing carotene far in excess of the vitamin A content but it will not remove the last traces of carotene or xanthophylls from vitamin A solutions.

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THE DIRECT COLORIMETRIC DETERMINATION OF UREA IN BLOOD AND URINE

By S. B. BARKER

(From the Department of Physiology, College of Medicine, University of Tennessee, Memphis)

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Methods for the determination of urea in biological fluids have passed through an interesting cycle (*cf.* Peters and Van Slyke (3) pp. 539-544). The earliest procedures were strictly chemical ones, involving precipitation of the urea as an insoluble mercury complex or decomposition of the urea by means of heat or hypobromite. The use of the highly specific enzyme urease occurring in the jack and soy bean, introduced in 1913 by Marshall, has been the foundation of all generally accepted procedures since that time, although heat and hypobromite are still occasionally employed clinically as being more convenient than urease. The most widely used modification is that in which the ammonia formed by enzymatic action is aerated into acid and titrated or nesslerized. Many attempts have been made to work out a simpler procedure with direct nesslerization of the urease-treated filtrate in order to avoid aeration; however, turbidity too often materializes to warrant the wide acceptance of direct nesslerization.

Fosse introduced xanthydrol as a reagent for precipitating urea, but this method has not gained wide usage, principally because of its lack of dependability. In 1942, Ormsby reported a technique for the direct colorimetric determination of urea itself, using diacetyl monoxime, a reagent employed by Fearon for the estimation of citrulline (2). Thus, the determination of urea has turned away from enzymatic methods and back to more strictly chemical ones. This change certainly involves a decrease in specificity, but at the same time a tremendous increase in convenience. Urease methods will remain the standards of reference, but need not be required for many ordinary purposes. The present paper describes a further modification of the diacetyl monoxime procedure, rendering it more uniform and convenient.

EXPERIMENTAL

Reagents—

Sulfuric acid. A 50 per cent by volume solution. This should be prepared by the cautious addition of 500 ml. of c.p. sulfuric acid to 500 ml. of distilled water in a Pyrex vessel. The solution should be thoroughly cooled after being mixed, transferred to a liter volumetric flask or to a graduated cylinder, and made to volume. This elaborate procedure eliminates gross

variabilities resulting from the volatilization of water from the hot mixture as the acid is being added.

Diacetyl monoxime. 3 per cent aqueous solution. This solution can be kept indefinitely if stored in a refrigerator when not in use. It is even stable for 4 weeks at room temperatures of 30–40°, after which time there are indications of changes in the solution.

Potassium persulfate. 1 per cent aqueous solution. Samples of this reagent have been kept in the refrigerator for as long as 8 weeks, and one solution for 6 weeks at elevated room temperatures, with no indication of deterioration. However, it would seem wise to renew the solution every 4 to 6 weeks, and to store it, when not in use, in a refrigerator.

Urea standards. A convenient stock standard is one which contains exactly 1 mg. of urea N per ml., obtained by dissolving 1.0717 gm. of desiccator-dried c.p. urea in distilled water and making the solution to 500 ml. A few drops of toluene or chloroform should be used as a preservative. This stock solution can be diluted to whatever concentration may be desired.

Protein Precipitation—The routine Folin-Wu tungstate or Somogyi's zinc procedures can be used (*cf.* (3) p. 65). If the latter is to be employed, the modification in which an acid zinc sulfate solution is used (4) is recommended. Trichloroacetic acid does not give satisfactory results.

Procedure

If the blood urea level is completely unknown, 1.0 ml. of a 1:10 protein-free filtrate is used plus 1.0 ml. of distilled water; if the level is known to be less than 75 mg. of urea N per 100 ml. of blood, 2.0 ml. of filtrate are used, without the addition of water. In urea clearance tests, the greater accuracy of the procedure with 2.0 ml. is highly desirable.

The 2.0 ml. of urea-containing solution are placed in a Klett-Summerson colorimeter tube,¹ followed by 0.25 ml. of the 3 per cent diacetyl monoxime solution. 4 ml. of the 50 per cent sulfuric acid are added, and the contents thoroughly mixed.

The tubes are placed in a suitable rack, and put into vigorously boiling water for 10 minutes. Glass marbles are used to cover the open ends of the tubes while they are being heated. At the end of the 10 minute heating period, 0.25 ml. of the 1 per cent persulfate solution is added to each tube not later than 5 minutes after the tubes are taken from the bath. Each tube should be shaken immediately after the persulfate is added to mix the contents thoroughly and quickly. The tubes are then left at room temperature.

¹ Or any convenient tube. In this laboratory, especially selected Pyrex test-tubes No. 9820 are used, since they have lips and are more rugged than the tubes supplied with the Klett-Summerson instrument.

The colorimeter reading on each tube should be made 15 minutes after the addition of persulfate. In this laboratory, the readings are made with a Klett-Summerson photoelectric colorimeter (5), with the Klett No. 42 blue filter. If the reading is higher than 500, representing more than 150 γ of urea N, the determination can be saved by adding 5.0 ml. of distilled water, mixing thoroughly, and reading. Such a dilution tested on standard solutions, urines, and uremic blood filtrates containing up to 250 γ has given, within 5 per cent, the same intensity of color as that produced by using one-half of the original aliquot. By this simple expedient, the range of the method can be extended by two-thirds, to 250 mg. per cent of urea N.

The procedure has been successfully applied to urine, without any necessity for the preliminary removal of ammonia with permutit. A convenient dilution for normal urines has been found to be 1:200 if 2.0 ml. are to be used, or 1:100 with 1.0 ml. If unusually dilute or concentrated

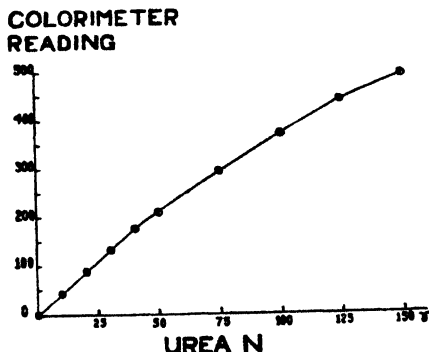


FIG. 1. Relationship between colorimeter reading and micrograms of urea N in the aliquot used.

urines are encountered, these dilutions must, of course, be altered correspondingly.

Results with Method—Fig. 1 shows the relationship obtained in this laboratory between micrograms of urea N and colorimeter readings (after the blank value for the reagent is subtracted). It will be noted that a straight line is obtained below 40 γ , enabling one to use a simple proportionality calculation for this lower portion. If amounts higher than 40 γ are encountered, the curve must be used. When the aliquot is 1.0 ml. of a 1:10 filtrate, the dilutions are such that the micrograms of urea N in the 1.0 ml. are also the milligrams of urea N per 100 ml. of blood. When 2.0 ml. of filtrate are taken, the value in micrograms must be divided by 2 in order to give mg. per cent. Amounts present in filtrates representing dilutions other than 1:10, in aliquots other than 1.0 or 2.0 ml., or in diluted urines can easily be calculated.

It is now generally recognized that any colorimetric procedure is more accurate if a photoelectric colorimeter is used instead of visual comparison of the colors; this is especially true for yellows. However, many readings of blood filtrate and urine colors have been performed in this laboratory with a visual colorimeter containing a blue filter. Provided that not less than 20 γ of urea N are present in the sample taken, clinically satisfactory results have been obtained. This necessitates the use of 2.0 ml. of filtrate for normal blood.

Proof of Method—The customary comparison and recovery tests have been conducted with this procedure. Table I summarizes the results obtained with human blood. As the reference, a well standardized urease procedure was used, in which the ammonia formed from the urea by the enzyme was diffused over into acid in a Conway unit and determined by nesslerization (1). Most of the results shown were obtained on the Folin-Wu tungstate filtrate; as can be seen from Table I, good agreement was obtained between the two procedures applied to filtrates covering a wide range of values. Furthermore, 5.0 to 100.0 mg. per cent of urea N added to various blood samples before protein precipitation were quantitatively recovered. The Somogyi zinc precipitation was also employed in many instances, and in general proved to be satisfactory. However, in several cases of very low urea levels, the zinc filtrate gave distinctly lower values than the tungstate, as indicated by the use of urease. No recoveries of added urea were made in these instances, and the cause of the low results is not known.

Table II shows that equally satisfactory results can be obtained with human urines. The values obtained with this diacetyl monoxime technique agreed well with those obtained when urease was used, and the recoveries were excellent. Inasmuch as ammonia does not react with the diacetyl reagent, urines do not need to be treated with permittit, but can be diluted and run directly. The pigment should be adequately disposed of by the 1:200 dilution; if not, permittit can be used for decolorization.

No attempt has been made in this study to check all the substances which might yield color with this procedure. Such complete lists are available for other procedures with a diacetyl monoxime reagent (*cf.* Ormsby (2)). It was thought quite adequate to determine the effects of only those substances likely to be encountered in blood and urine. Uric acid, creatine, creatinine, and several amino acids (glycine, alanine, glutamic acid, arginine, lysine, asparagine), all in amounts equivalent to 20 mg. of N per 100 ml. of blood, gave no color themselves and did not interfere with the color intensity of 20 mg. per cent of urea N. Neither ammonia equivalent to 50 mg. of N per 100 ml. of blood nor glucose (500 mg. per cent) reacted or interfered.

Allantoin, on the other hand, gave about 70 per cent as much color as urea when calculated on the basis of the two ureido nitrogens (a logical procedure, since the essential difference between allantoin, which reacts, and uric acid, which does not, is the presence in the former of the opened

TABLE I
Determination of Urea in Human Blood

Sample No.	Diacetyl monoxime method						Urease method
	Folin-Wu filtrate			Somogyi filtrate			Urea N
	Urea N	Added	Recovered	Urea N	Added	Recovered	
	mg. per cent	mg. per cent	per cent	mg. per cent	mg. per cent	per cent	mg. per cent
1	5.2			4.5			5.7
2	6.3	10.0	96	5.2			6.9
3	6.3			5.1			6.4
4	7.1			6.0			7.0
5	7.2	15.0	95	6.8	15.0	99	6.8
6	7.6			6.1			8.0
7	10.1	10.0	98	9.2	10.0	97	9.8
8	10.1			9.4			9.9
9	10.7	15.0	102	10.7	15.0	92	10.6
10	11.1	100.0	98				10.9
11	11.2	10.0	98	10.6	10.0	85	11.3
12	12.2	10.0	93	11.4	10.0	95	12.3
13	14.0			13.7			14.6
14	16.1	5.0	105	15.2	5.0	106	16.3
15	17.3	30.0	95	14.2			17.8
16	17.8	10.0	97	18.1	10.0	95	17.9
17	18.8	10.0	100				18.4
		20.0	96				
18	21.4	50.0	102	21.2	50.0	99	22.0
19	33.2			32.8			33.4
20	45.5			45.5			45.2
21	50.5			50.0			51.6
22	51.7						51.4
23	51.8						53.6
24	54.0						53.2
25	57.9						57.0
26	62.2	50.0	100	61.7	50.0	98	61.3
27	71.1			69.4			71.0
28	76.0			77.2			74.0
29	116.0			116.0			113.5
30	316.0	25.0	97	315.5	25.0	96	302.5

ureido chain). This is of no concern for human blood or urine. However, in the analysis of blood or urine from animals with a high uricolytic index, such as the dog, the colorimetric values should represent urea plus allantoin. From Tables II and III, it can be seen that the diacetyl monoxime method

applied to dog blood and urine has been found to give consistently more "urea N" than the urease technique. In the case of the urines, the differences are small (about 5 per cent), and may well represent allantoin. However, on the tungstate filtrates this difference was 1 to 6 mg. of "urea N" per 100 ml. of blood, averaging 3.7 mg. per cent, as compared with the average true urea N of 12.4 mg. per cent (about 30 per cent). When this value is multiplied by the factor 8.3 (color from 1.00 mg. of urea N = color from 1.47 mg. of allantoin ureido N = 8.30 mg. of allantoin), an average of

TABLE II
Determination of Urea in Human and Dog Urine

Sample No.	Human				Dog			
	Diacetyl monoxime method			Urease method	Diacetyl monoxime method			Urease method
	Urea N	Added	Recover- ed	Urea N	Urea N	Added	Recover- ed	Urea N
	mg. per 100 ml.	mg. per 100 ml.	per cent	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	per cent	mg. per 100 ml.
1	105	200	97	109	25			25
2	275	50	97	281	35			32
3	374			381	126	50	96	124
4	431	200	99	428	268	100	101	261
5	438			430	452	200	95	434
6	463	100	96	477	754			728
7	480	200	96	490	785	100	99	763
8	485	100	102	476	2932	500	97	2993
9	513	200	101	525	6618			6347
10	561	50	95	548	7204	200	96	6843
11	587			603				
12	589	100	98	580				
13	725			731				

31 mg. per cent of allantoin is obtained. This is so high as to cast considerable doubt on interpretation of the difference as allantoin.

It will be noted from Table III that zinc filtrates usually gave values for dog blood lower than did tungstate, but still considerably above the true (urease) values. In following this up, we precipitated blood proteins by a variety of other reagents, but without finding any improved filtrate. Even the use of plasma instead of whole blood did not alter the results. The only procedure which was found to yield values close to the true ones involved the treatment of the Somogyi filtrate with permutit. In the experiments listed in the lower portion of Table III, 7.0 ml. of zinc filtrate were shaken 5 to 10 minutes with 1 gm. of permutit² and filtered. The

² Although the permutit used was labeled "Prepared according to Professor Otto Folin," it was found necessary to wash it several times with distilled water to remove a fine, slowly settling material. The remaining permutit was oven-dried for use.

zinc filtrate values, previously 15 to 45 per cent too high, were brought down to -5 to +8 per cent of the urease figures by this procedure. That this decrease was not due to the removal of urea is supported by the

TABLE III
Determination of Urea in Dog Blood

Sample No.	Diacetyl monoxime method							Urease method
	Folin-Wu filtrate			Somogyi filtrate				Urea N
	Urea N	Added	Recover-	Urea N	Added	Recover-	Permutit	
	mg. per cent	mg. per cent	per cent	mg. per cent	mg. per cent	per cent	mg. per cent	mg. per cent
1	11.8	15.0	106	11.6	15.0	98		8.9
2	12.8							11.5
3	13.5							12.2
4	14.2	15.0	103	13.2	15.0	101		10.4
5	15.3	10.0	98					14.0
6	15.5	15.0	101	14.3	15.0	100		10.1
7	15.6	10.0	96	14.1	10.0	99		13.0
8	16.5	15.0	97	14.1	15.0	98		10.0
9	17.4			15.6				13.6
10	18.3			16.6				14.1
11	18.8	10.0	95	16.9	10.0	97		14.1
12	19.3			17.0				15.0
13	21.2			19.2				15.9
Average...	16.2		100	15.3		99		12.5
1	9.8			9.8			8.7	9.2
2	11.5			11.7			9.5	9.4
3	11.8*			11.6			9.4	8.9
4	13.1			13.0			11.2	11.0
5	14.2*			13.2			10.5	10.4
6	15.5*			14.3			10.1	10.1
7	16.5*			14.1			9.7	10.0
8	16.5			15.6			13.1	12.1
9	22.0			21.2			15.7	15.0
10	22.2			21.8			18.0	16.8
Average...	15.3			14.6			11.6	11.3

* These values are duplicated in the upper portion of the table.

quantitative recovery of urea added to the filtrate before treatment with permutit.

Spectrophotometric study, with a Beckman quartz spectrophotometer, was undertaken in an attempt to obtain further information on the source of the extra color yielded by filtrates of dog blood. As can be seen from

Figs. 2 and 3, the absorption curves of human and dog urines and of human blood filtrates correspond very closely to the standard urea curves. In contrast, the dog blood filtrates show considerable deviation from pure urea in the region of 400 to 450 $m\mu$ (and also from 500 to 550 $m\mu$, although this region is not transmitted by the blue filter used), especially marked when the Folin-Wu filtrate is used. Curves for allantoin and citrulline are included, the latter taken from Ormsby (2). The absorption characteristics of the allantoin color so closely resemble those of the color with urea as to

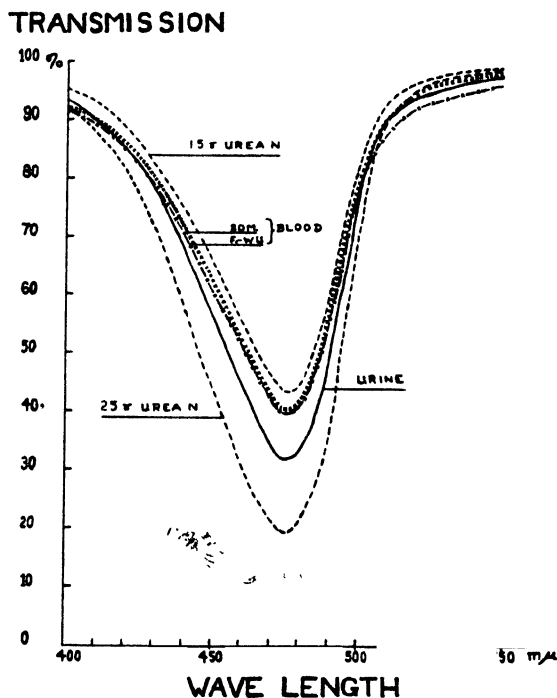


FIG. 2. Absorption characteristics of the diacetyl monoxime color with human urine and blood filtrates.

make even qualitative identification in a mixture impossible; this combines well with the reason previously advanced for not considering allantoin responsible for much of the difference between colorimetric and urease values actually found with dog blood. If citrulline were present, considerable flattening of the typical absorption peak at 476 $m\mu$ would be expected. Since this was not found with the filtrates, citrulline can probably be ruled out. Study of the permutit-treated zinc filtrates revealed only a non-specifically decreased absorption all along the curve.

DISCUSSION

Analytical Precautions—Although the procedure has been simplified to the point of uniform use of 2.0 ml. of aqueous urea solution and 4.0 ml. of 50 per cent sulfuric acid, 1.0 ml. of filtrate can be used with 5.0 ml. of 40 per cent acid. Logically, this relationship could be carried out to 4.0 ml. of filtrate and 2.0 ml. of concentrated sulfuric acid, if very dilute urea solutions were to be encountered, or to give a deep color for visual colorimetry. This can be done, but the results are extremely erratic, because the addition

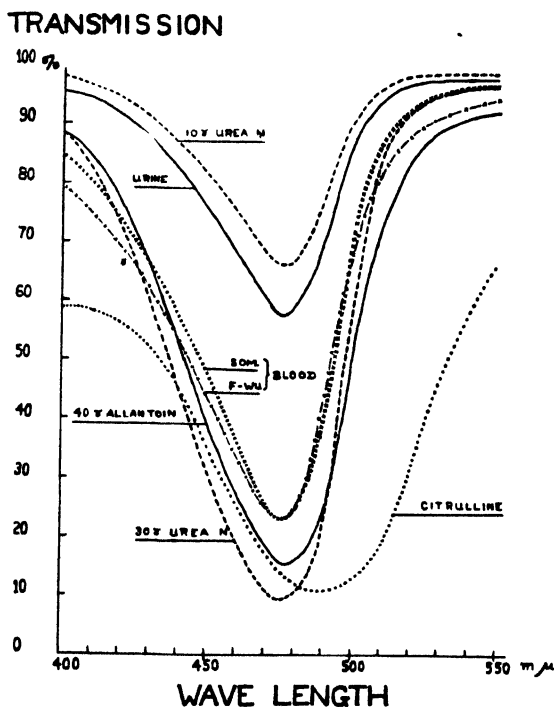


FIG. 3. Absorption characteristics of the diacetyl monoxime color with dog urine and blood filtrates.

to water of sulfuric acid any stronger than 50 per cent evolves so much heat that the 10 minute heating period at 100° cannot be judged with any accuracy. Cooling could probably be resorted to, but was considered to introduce an unjustified complication.

As indicated under "Procedure," the period of heating at 100° should be 10 minutes. $\frac{1}{2}$ minute on either side of this does not significantly alter the results; longer heating produces an undesirable darkening. As Ormsby noted, the tubes must be kept out of any direct sunlight during and after

the heating. Such exposure causes a premature development of the color, followed by a rapid fading. In fact, the determination is best carried out in a part of the laboratory definitely removed from windows, to avoid even indirect glare. There is, however, no advantage in keeping the tubes in complete darkness.

The addition of the persulfate to the hot solution is advantageous in that maximum color development is achieved within 15 minutes for all amounts of urea. If the tubes are thoroughly cooled before the addition of persulfate, the time course of color development varies widely, according to the amount of urea present. It has been found unnecessary to control the actual temperature of the tubes at the time of persulfate addition, provided this is done within 5 minutes after removal from the water bath. The tubes ordinarily are still warm at the end of the 15 minutes, when the colors are to be read. If there appears to be any particular reason for desiring cool solutions, the tubes may be placed in cool tap water during the 12 to 14 minute interval without altering the results.

After the maximum color development is reached, fading becomes noticeable to the extent of about 0.3 γ per 5 minutes, the same in blood filtrates and urines as in standard solutions. The percentage error caused by this fading depends, of course, on the total amount of urea N present. With 20 γ , from 2.0 ml. of a 1:10 filtrate of normal blood, 0.3 γ represents an error of -1.5 per cent. It will readily be seen that an extra 15 minute delay in reading the colors would produce less than 5 per cent error. If the blood urea N level is elevated, the error will be correspondingly less. The number of determinations that can be performed in one series with only a 5 per cent error due to fading thus depends upon the analyst's ability to add persulfate to all tubes within the 5 minute limit and opportunity to read all colors between 15 and 30 minutes later. The maximum number will usually be found to be about twenty single analyses, or ten in duplicate, when a photoelectric colorimeter is available. If a visual colorimeter must be used, the readings are slower, but this is compensated for by the fact that the standards will fade along with the unknowns. For the most accurate results with this procedure, a definite routine should be worked out for the entire process from the time of addition of the acid to the reading of the colors. If the steps involved are varied too widely, the results obtained can be expected to be erratic.

Preservatives—If blood filtrates or urines are not to be analyzed promptly after they have been obtained, they should be stored in a refrigerator after the addition of a drop of toluene or chloroform. Thymol interferes with the reaction, and must not be used.

The advantages of a direct colorimetric method for the determination of urea are apparent to anyone who has used urease procedures, especially

when only small amounts of blood are available. The present method differs from that devised by Ormsby in three principal ways: (1) the use of a non-volatile acid instead of concentrated HCl, (2) a need for only 1.0 or 2.0 ml. of filtrate for each determination, instead of 3.0, and (3) the addition of persulfate to the hot solution to produce quick and uniform color development from all amounts of urea tested. It should also be noted that the range of the method, 10 to 150 mg. per cent of urea N, broad as it is, can be extended to 250 mg. per cent simply by the addition of 5.0 ml. of distilled water to the final solution. This covers practically all values encountered.

SUMMARY

1. A direct colorimetric procedure has been described for the quantitative determination of urea in blood and urine. The urea is condensed with diacetyl monoxime in the presence of sulfuric acid, and the resulting color enhanced by the addition of potassium persulfate.

2. 10 to 150 γ of urea N can be determined, corresponding to 10 to 150 mg. of urea N per 100 ml. of blood. By a supplementary dilution, this range can be extended to 250 mg. per cent if necessary.

3. The method has been found to give the same results on human blood filtrates and urines as those by urease. The colorimetric results on dog urine also are very close to the urease values, but, in the case of dog blood, this procedure indicates 1 to 6 mg. per cent more "urea N" than the urease method shows to be present. This discrepancy can largely be removed by treating the Somogyi zinc filtrate with permutit. This modification is entirely empirical, since the extra color cannot be attributed to any specific substance.

The author wishes to acknowledge gratefully the work of Miss Hortense S. Louckes on the charts.

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GENERAL CHARACTERISTICS OF THE PARTIAL HYDROLYSIS PRODUCTS FROM THE ACTION OF PROTEOLYTIC ENZYMES ON CASEIN

By THEODORE WINNICK

(From the Department of Chemistry, University of Idaho, Moscow)

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The recent study by Gordon, Martin, and Synge (1) of the partial acid hydrolysis products of gelatin illustrates the value of this type of approach to the problem of protein structure. These investigators used electro-dialysis to obtain basic and neutral fractions from the partially hydrolyzed protein, and then by the use of chromatographic adsorption were able to isolate from the neutral fraction the acetyl derivatives of a number of peptides.

In the present study of the digestion products of casein, proteolytic enzymes have been employed as hydrolytic agents. Synge (2) points out that simultaneous synthetic reactions may complicate the characterization of enzymic hydrolytic products. While this possibility of peptide bond synthesis cannot be dismissed, it is significant that certain physicochemical properties of the digestion products are very similar and reproducible when samples of a given protein are digested by different proteolytic enzymes. Although the final peptide-amino acid mixture may represent the net effect of hydrolytic and synthetic reactions, it appeared of interest to compare these non-protein products from the following standpoints: free amino acid content, number of amino acid residues in the average molecule, behavior upon electrodialysis, specific rotation, percentage of nitrogen, and average molecular weight.

Also, the products from short periods of protein digestion were compared with those obtained by prolonged protease action. The purpose of this comparison was to test further a recent theory of the nature of protein cleavage, formulated by Tiselius and Eriksson-Quensel (3). These investigators found that the non-protein products formed during the digestion of ovalbumin by pepsin had an average molecular weight of 1080, as measured by electrophoretic, sedimentation, and diffusion methods. Since there were no significant amounts of products of relatively high molecular weight, they concluded that, in the process of proteolysis, relatively few protein molecules are split very rapidly in each time interval, rather than that a general slow cleavage of all of the molecules occurred simultaneously. This view is supported by the observation of Haugaard and Roberts (4) that the ratio of amino to total nitrogen of the non-protein products remains con-

stant throughout the digestion of lactoglobulin by pepsin. This nitrogen ratio, and also the specific rotation, of the digestion products is determined in the present study for both the initial and late stages of the hydrolysis of casein by different proteases.

EXPERIMENTAL

Materials—Casein was used as the substrate in all protease digestions because of its high digestibility and high specific rotation. The protein was prepared by the method of Van Slyke and Baker (5). The white product contained 15.4 per cent nitrogen, calculated on an ash- and moisture-free basis. The enzymes were crystalline preparations, except the papain, which was Merck's variety. The activity of the enzymes was not assayed quantitatively, but their potency is indicated by the fact that the weight of casein was 100 to 200 times that of the proteases used in the digestions.

Digestions—The details for each series of digestions are given in Tables I to III. In the experiments described in Table I, tests on samples of the final digests indicated that the conversion of protein to non-protein material was 95 to 100 per cent complete. This was judged from measurements of the percentage of the total nitrogen which was soluble in 5 per cent trichloroacetic acid solution. The casein-pepsin digestion was not quite as complete as the others, since about 10 per cent of the protein was still precipitable by trichloroacetic acid after 36 hours.

In Table II completion of the peptidase action was not attempted, while in Table III the products are from only the initial stages of digestion.

Total Nitrogen—Aliquots of solutions were analyzed by the micro-Kjeldahl method.

Amino Nitrogen—The Van Slyke nitrous acid method was used to determine the sum of the amino nitrogen of peptides plus that of free amino acids. 1 or 2 ml. samples of solutions were analyzed. In agreement with the observation made by Pope and Stevens (6) for the tryptic digestion of fibrin, a shaking time of 16 to 20 minutes at about 25° was found necessary for complete (maximum) evolution of nitrogen gas. Close agreement was obtained when the same solutions were analyzed for amino nitrogen by the copper method of Pope and Stevens.

Carboxyl Nitrogen—The α -amino nitrogen of free amino acids was determined by gasometric measurement of the equivalent amount of carbon dioxide evolved from the proximal carboxyl groups, according to the Van Slyke method (7). 1 or 2 ml. samples were heated with ninhydrin at pH 2.5 in all-glass reaction vessels, and analyzed on the micro scale. Duplicate determinations agreed to within 1 to 2 per cent.

Electrodialysis—Following the digestion of a casein solution with a protease (Table I), the mixture was heated to 100°, cooled, adjusted to

about pH 7.5 with 6 N HCl or NaOH, and filtered, if necessary, to remove any undigested protein residue. About 20 ml. of filtrate were saved for nitrogen analyses. The rest of the filtrate was subjected to electrodialysis in the five-chambered glass Lödösöl apparatus (Central Scientific Company). The compartments were separated by sheets of heavy cellophane, with rubber rings between the glass edges and the cellophane. The apparatus was assembled with the large chamber (ordinarily the center one) set at one end as the terminal anode compartment. The digest was placed in the chamber next to the compartment which contained the cathode; *i.e.*, next to the chamber at the other end of the apparatus. Thus the assembly consisted of three anode compartments, the digest-containing compartment, and one cathode compartment. All chambers (except the one containing the digest) were filled with distilled water. The maximum removal of electrolytes with minimum migration of peptides resulted with this arrangement.

With an ammeter and a 100 watt lamp in series, a 110 volt direct current was passed through the apparatus for 12 to 13 hours. The casein digest was stirred continually during this time. Cooling of the solutions was not necessary. At 2 to 3 hour intervals most of the contents of each chamber (except the one with the digest) were replaced with distilled water. The ammeter reading rose to 0.2 to 0.3 ampere in 3 to 4 hours, and then fell gradually to about 0.02 ampere at the end of 10 to 11 hours. The pH of the digest changed gradually in an acid direction, reaching the final values given in Table I.

During the later part of the electrodialysis, all chambers except the end anode compartment gave positive biuret and phenol tests. The solution in the end anode chamber contained no organic nitrogen, owing perhaps to its very acid pH (about 2). Since this solution gave negative tests for nitrate and nitrite with diphenylamine in sulfuric acid, it appeared unlikely that oxidation of amino acids or peptides had occurred at the anode.

At the end of the electrodialysis period, the casein digest was removed and concentrated overnight in a vacuum desiccator (at room temperature) to approximately its original volume. This compensated for the dilution which occurred during the electrodialysis. The digest was filtered through filter paper of fine porosity to give a perfectly clear solution.

Nitrogen Content of Electrodialyzed Material—A 5 ml. aliquot of the filtered solution was evaporated to about 0.2 ml. in a porcelain crucible, and then several ml. of absolute ethyl alcohol added to flocculate the organic material. The alcohol and water were evaporated off, and the white residue was dried to constant weight at 90–100°. This residue contained about 1 per cent of ash. Its nitrogen content (corrected for ash) was calculated with the aid of the Kjeldahl nitrogen content of the original solution.

Average Molecular Weight of Electrodialyzed Material—The freezing point of the solution was compared with that of distilled water, the Beckmann apparatus being used. The amino acids and peptides remained completely soluble at the freezing points of the solutions. Duplicate determinations usually agreed to within 0.01° for depressions of approximately 0.2° . The gm. of solute per 100 gm. of water were given with sufficient accuracy by the percentage of solids in the solution, determined according to the preceding paragraph. The molecular weight, reproducible to within about 5 per cent, is given by

$$\frac{1.86 \times \text{gm. of solid per liter of solution}}{\text{Observed freezing point depression}}$$

An approximate calculation, based on the ash content of the dry organic material, suggests that the small quantity of electrolytes still remaining after electrodialysis would be responsible for a freezing point lowering of about 0.02° in the solutions used. Hence, the calculated molecular weights would be approximately 10 per cent greater if a correction could be made for this ionic effect.

Specific Rotation—Optical rotation was measured at about 25° with a General Electric sodium vapor lamp as the source of illumination. It has been shown (8) that variations in temperature and pH have very little effect on the optical rotation of enzymic digests of casein. The observed rotations were of the order of $3-5^{\circ}$ in a 1 dm. tube. Duplicate measurements agreed to within $0.02-0.05^{\circ}$. The specific rotations were calculated with the aid of the values for the gm. of organic solid per 100 ml.

DISCUSSION

Table I compares the action of five different proteases on casein. After prolonged digestion, the amino nitrogen of the filtered solutions constituted 14 to 19 per cent of the total nitrogen. However, in order to express the *increase* in amino nitrogen due to proteolysis, these figures must be corrected for the free amino groups (due to lysine) in casein. Analyses by the nitrous acid method with a reaction time of 18 minutes indicated that the free amino nitrogen constituted 5.2 per cent of the total nitrogen in the intact casein molecule, in good agreement with the value obtained by Van Slyke and Birchard (9). When 5.2 was subtracted from the values in Column 3 of Table I, the increases in amino nitrogen amounted to from 8.8 to 14.0 per cent of the total nitrogen. The ratios of the amino nitrogen liberated by proteases to the amino nitrogen freed by complete acid hydrolysis (67 per cent of the total nitrogen¹) gives the fraction of the total peptide bonds

¹ This value was derived from nitrogen analyses on samples of the electrodialyzed digests, following complete hydrolysis of the latter with 6 N hydrochloric acid. Ac-

split. These ratios, ranging from 0.13 to 0.21, indicate that the average molecules (including free amino acids) were approximately pentapeptides in the chymotrypsin, ficin, and papain digests, and heptapeptides in the pepsin and trypsin digests. Tiselius and Eriksson-Quensel have concluded that the peptic digestion products of ovalbumin have an average of 8 amino acid residues in the peptide chains.

Values for carboxyl nitrogen in Column 4 of Table I show that 1 to 3 per cent of the total nitrogen was in the form of free amino acids. The results

TABLE I
*Characteristics of Partial Hydrolysis Products Formed by Action of Different
Proteases on Casein*

Approximately 30 mg. of protease, in 5 ml. of water, and 100 ml. of 6 per cent casein solution were employed in each series. The casein was dissolved in an acetic acid-hydrochloric acid mixture for the digestion with pepsin. For digestions with the other proteases, the casein was dissolved in 0.1 N NaOH, and then adjusted with 6 N H_3PO_4 to the desired pH. The papain was dissolved in 0.05 M NaCN (adjusted to pH 7) instead of in water. The ficin did not require activation. The enzyme-substrate mixtures were incubated for about 36 hours at 40°, with small amounts of toluene preservative.

Protease	Initial pH of casein sub- strate	Amino N in dige- stion prod- ucts	Car- boxyl N in dige- stion prod- ucts	Electrodialysis			Digestion products remaining after electrodialysis				
				Final pH	N mi- grated toward anode	N mi- grated toward cath- ode	Amino N	Car- boxyl N	N in dry mate- rial	Aver- age mol. wt.	Specific rota- tion, [α] _D ²⁵
				(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
(1)	(2)	(3)	(4)		per cent of total N	per cent of total N	per cent of total N	per cent of total N	per cent		degrees
Chymo- trypsin.	9.0	17.7	3.1	5.6	2.0	4.8	15.2	2.1	14.3	600	-95
Trypsin...	9.0	14.7	1.1	6.0	1.3	3.5	15.8	1.5	13.9	560	-89
Pepsin....	1.4	14.0	1.0	5.8	0.1	0.9	13.1	0.5	14.0	590	-85
Ficin.....	7.3	18.9	1.0	5.8	2.1	5.7	17.3	0.8	14.0	480	-87
Papain...	7.5	19.2	2.3	5.9	1.9	6.0	18.0	2.1	14.1	450	-78

with trypsin agree with those reported by Van Slyke, Dillon, MacFadyen, and Hamilton (7). Haugaard and Roberts, also using the ninhydrin-

cording to the nitrous acid method, the amino nitrogen (corrected for amino nitrogen in the intact protein) constituted 69 to 70 per cent of the total nitrogen, while the corresponding value obtained by the ninhydrin method was 64 to 65 per cent. This difference may be due in part to the fact that the former method, but not the latter, includes most of the amide nitrogen of casein. However, it may be noted that the ninhydrin method, but not the nitrous acid method, includes the nitrogen of proline and hydroxyproline.

carbon dioxide method, found free amino acids to the extent of 1 to 2 per cent of the total nitrogen in peptic digests of lactoglobulin.

The ratios of carboxyl nitrogen to the amino nitrogen freed by complete acid hydrolysis indicate that from 1.5 to 4.5 per cent of the total amino acids of casein was liberated by protease action. It is interesting that the carboxyl and amino nitrogen values with crude papain do not differ significantly from those with crystalline ficin and chymotrypsin.

Following electrodialysis, the pH of the digests was between 5.6 and 6.0 in the five cases. Probably the isoelectric points of the majority of the peptides were in this region. The neutral character of the bulk of the peptides is indicated by their failure to migrate. The nitrogen lost by migration of amino acids and peptides toward the cathode and anode ranged from 1.0 to 7.9 per cent of the initial amount.

The percentages of amino and carboxyl nitrogen were not altered significantly by the electrodialysis procedure. Except in the case of the trypsin digest, the values were slightly lower, perhaps reflecting the migration of smaller peptides and free amino acids. Also the values would be slightly modified by migration of acidic and basic amino acids.

The average nitrogen contents of the (dried) electrodialyzed peptide-amino acid mixtures were somewhat lower than the nitrogen content of the original casein (15.4 per cent nitrogen), owing presumably to the addition of water during protein hydrolysis.

If the weight of the average amino acid residue of casein is taken as approximately 115 (10), the molecular weight values for the electrodialyzed materials imply that the average molecules (including free amino acids) were about pentapeptides in the chymotrypsin, trypsin, and pepsin digests, and tetrapeptides in the ficin and papain digests. Hence it appears that the chain lengths are somewhat shorter when calculated on the basis of molecular weights, as compared with the corresponding estimates derived from nitrogen analyses. This discrepancy is probably due in part to the small amounts of inorganic electrolytes present. As previously indicated, the resulting ionic effects would increase slightly the observed freezing point depressions, and hence give slightly lowered values for the molecular weight.

The specific rotations in Table I were lower than that obtained for the original casein, $[\alpha]_D^{25} = -103^\circ$ at pH 8.0. Almquist and Greenberg (11) found $[\alpha]_D^{25} = -100^\circ$ for casein at pH 7.5. The specific rotation of the papain digest was approximately the same as the values reported (8) for digestion products from the action of several different plant proteases on casein solutions.

The action of a peptidase upon the split-products from digestions with proteases is indicated in Table II. The quantity of carboxypeptidase used was relatively large compared with the amount of substrate (1:33). How-

ever, most of the enzyme precipitated during the digestions as the pH changed from 9.0 to about 8.5, and was removed subsequently by filtration. The amino and carboxyl nitrogen values are similar to those reported for digestions with crude trypsin (6). If the amino nitrogen values are expressed as increases (*i.e.* corrected as before for the terminal amino nitrogen of lysine), and compared with the carboxyl nitrogen values, it is found that slightly more than half of the molecules in each digest are free amino acids.

TABLE II
Action of Carboxypeptidase on Protease Digestion Products

20 ml. portions of three of the electrodyalyzed mixtures described in Table I were adjusted to pH 9.0 with 6 N NaOH, treated with a suspension of approximately 30 mg. of carboxypeptidase in 1 ml. of water, and incubated for about 24 hours at 40°.

Substrate; casein digested by	Amino N	Carboxyl N	Specific rotation, $[\alpha]_D^{25}$
	<i>per cent of total N</i>	<i>per cent of total N</i>	<i>degrees</i>
Chymotrypsin.....	27.3	11.9	-83
Trypsin.....	29.5	12.5	-79
Papain.....	36.0	16.8	-68

TABLE III
Characteristics of Hydrolytic Products Following Partial Digestions of Casein by Different Proteases

To 20 ml. portions of 6 per cent casein, prepared as described in Table I, were added 5 mg. of enzyme in 1 ml. of water (0.05 M NaCN in the case of papain). After 5 to 10 minutes at 45-50°, 5 ml. of 20 per cent trichloroacetic acid were added to each digestion mixture, and the precipitate of undigested casein removed by filtration. Analyses were made upon the filtrates. In calculation of the specific rotations, it was assumed that the non-protein substances contained 14.0 per cent nitrogen.

Protease	pH of substrate	Amino N	Specific rotation, $[\alpha]_D^{25}$
		<i>per cent of total N</i>	<i>degrees</i>
Chymotrypsin.....	8.8	13.6	-96
Trypsin.....	8.8	15.0	-89
Pepsin.....	1.5	14.5	-93
Ficin.....	7.2	13.8	-93
Papain.....	7.2	13.6	-90

However, the ratio of carboxyl nitrogen to the amino nitrogen freed by complete acid hydrolysis indicates that only 18 to 25 per cent of the total amino acids of the casein was liberated. These facts confirm the generally accepted view that peptidase action consists chiefly in the splitting of free amino acids from the ends of peptide chains, rather than the cleavage of polypeptides into smaller peptide molecules.

The lower specific rotations in Table II are indicative of further hydroly-

sis, since it has been shown (8) that the optical rotation decreases uniformly with increase in amino nitrogen during the hydrolysis of casein by different proteases and peptidases.

Table III gives the ratios of amino to total nitrogen and the specific rotations of the initial products from short periods of digestion of casein by the different proteases. Only 5 to 10 per cent of the nitrogen of the substrates was converted to non-protein nitrogen in these incomplete digestions. Comparison of the values in Table III with the corresponding data in Table I shows that no great changes in the amino nitrogen or specific rotation of the non-protein products occurred between the initial and the final stages of the digestions. The largest variations were in the digestions with ficin and papain. The results seem to support fairly well the view of Tiselius and Eriksson-Quensel, and Haugaard and Roberts, that relatively few protein molecules are split rapidly in each time interval in enzymic hydrolysis.

SUMMARY

A study was made of the partial hydrolysis products from the action on casein of the enzymes pepsin, trypsin, chymotrypsin, ficin, papain, and carboxypeptidase.

Following digestions with proteases, the average non-protein molecules contained from 5 to 7 amino acid residues, with 1.5 to 4.5 per cent of the total nitrogen in the form of free amino acids. After the removal of inorganic electrolytes in electrodialysis, some additional characteristics of the digestion products were determined, including the nitrogen content, specific rotation, and average molecular weight.

The further action of carboxypeptidase on protease digests of casein was interpreted as the splitting of free amino acids from the ends of polypeptide chains.

The initial products from short periods of protein digestion were compared with those from prolonged protease action. The specific rotations and ratios of amino to total nitrogen of the products did not differ significantly in these two cases, in agreement with a recent speculation on the mechanism of proteolysis.

The author wishes to thank the following for supplies of crystalline enzymes: Dr. M. L. Anson for the carboxypeptidase, Dr. R. M. Herriott for the pepsin, Dr. M. Kunitz for the trypsin and chymotrypsin, and Dr. D. M. Greenberg for the ficin.

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LETTERS TO THE EDITORS

THE EFFECT OF POLYAMINES ON BACTERIOSTASIS BY 4,4'-DIAMIDINODIPHENOXYPROPANE

Sirs:

Silverman and Evans¹ recently showed that the bacteriostatic effect of atabrine for *Escherichia coli* was decreased by presence in the medium of polyamines such as spermine, spermidine, tetraethylenepentamine, and triethylenetetramine. The effect of certain of these compounds on bacteriostasis by 4,4'-diamidinodiphenoxyp propane,² a drug ("propamidine")³ which is also effective in certain protozoal and bacterial infections,⁴ was determined.

The basal medium used for this investigation has been described;⁵ it was supplemented with 1 mg. of asparagine, 1 γ of *p*-aminobenzoic acid, and 3 γ of pyridoxine per 10 cc. The effects of adding various combinations of "propamidine diisethionate" and polyamines on the growth of *Lactobacillus casei* and *Streptococcus lactis* are shown in the table. Several conclusions may be drawn. "Propamidine" inhibits growth of these bacteria; sensitivity of various organisms to it varies considerably. Growth inhibition by it is largely prevented by simultaneous additions of certain polyamines to the medium. For *Lactobacillus casei*, triethylene-tetramine is most effective in this respect; tetraethylenepentamine and spermidine suppress growth before any action antagonistic to that of "propamidine" appears. For *Streptococcus lactis*, spermidine is non-toxic at levels effective in preventing bacteriostasis. Tetraethylene-pentamine is less effective in preventing bacteriostasis than is spermidine, and itself shows slight inhibitory action. It is much more effective in preventing bacteriostasis by "propamidine" than triethylenetetramine, which is only slightly active in this respect for this organism. This effect of polyamines is evident only in a limited range of concentrations. When large amounts of "propamidine" are added, it becomes irreversibly toxic.

¹ Silverman, M., and Evans, E. A., *J. Biol. Chem.*, **150**, 265 (1943).

² Ashley, J. N., Barber, H. J., Ewins, A. J., Newbery, G., and Self, A. D. H., *J. Chem. Soc.*, 103 (1942).

³ This drug was kindly furnished by Dr. A. J. Ewins of May and Baker, Ltd., in the form of its di-(β -hydroxyethane sulfonate) under the proprietary name "propamidine diisethionate." Dr. E. A. Evans, Jr., kindly furnished a sample of spermidine.

⁴ Thrower, W. R., and Valentine, F. C. O., *Lancet*, **244**, 133 (1943).

⁵ Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, **143**, 519 (1942).

Growth of some organisms (e.g. *Lactobacillus arabinosus*) is inhibited only when much higher concentrations of "propamidine" are used than are necessary to inhibit *Lactobacillus casei*. Preliminary results indicate

Effect of Polyamines on Bacteriostasis by 4,4'-Diamidinodiphenozopropane

<i>Lactobacillus casei</i>			<i>Streptococcus lactis</i> R		
"Propamidine diisethionate" added	Polyamine added	Galvanometer reading*	"Propamidine diisethionate" added	Polyamine added	Galvanometer reading*
mg. per 10 cc.	mg. per 10 cc.		mg. per 10 cc.	mg. per 10 cc.	
0	0	68	0	0	54
0.4	0	25	0.07	0	26
0.7	0	0	0.10	0	16
0	1 TT	78	0	10 TT	54
0	10 "	67	0.07	10 "	37
0.4	1 "	58	0.10	10 "	23
0.7	1 "	4	0	3 TP	42
0.7	10 "	54	0.07	3 "	50
0	3 TP	3	0.10	3 "	41
0	1 SP	1	0	1 SP	55
			0.07	1 "	53
			0.10	1 "	50

TT = triethylenetetramine; TP = tetraethylenepentamine; SP = spermidine phosphate. All solutions were neutralized with hydrochloric acid before being added to the medium.

* Uninoculated medium reads 0; a reading of 100 indicates no light was transmitted. *Lactobacillus casei* was incubated at 37° for 24 hours; *Streptococcus lactis* was incubated at 30° for 16 hours.

that inhibition of such organisms is not prevented by the polyamines so far tested.

*The University of Texas, Biochemical Institute
and the Clayton Foundation for Research
Austin*

ESMOND E. SNELL

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EFFECT OF ACID HYDROLYSIS ON THE ACTIVITY OF POLYSACCHARIDES IN THE ENZYMATIC SYNTHESIS OF STARCH*

Sirs:

It has been reported that the synthesis of polysaccharides through the action of purified phosphorylase preparations from potatoes¹ or muscle² requires the presence of natural starch or glycogen. These polysaccharides have in common branched glucosidic linkages. Their efficacy as activators of phosphorylase has been attributed to this fact.³ This would seem to be plausible since polysaccharides formed *in vitro* by potato and muscle phosphorylase and which appear to be straight chain molecules^{4,5} show no activating effect.

It occurred to us that the slight solubility of synthetic starch might account, in part, for its inability to act as an activator and that partial hydrolysis with acid might yield active fragments.

To study this question 0.125 gm. of the starch to be tested was suspended in 9.0 ml. of water in a 25 ml. volumetric flask. Cold concentrated hydrochloric acid was added to the mark, and the contents quickly mixed. The temperature was maintained at 27–28°. At intervals, 1 ml. of the hydrolyzing solution was removed and quickly added to sufficient potassium hydroxide (about 1.65 N) to bring the pH to 6.0. The neutralized solution was diluted to 10 ml. and 1 ml. of this (equivalent to 0.5 mg.) was used in place of the usual starch in an activity determination essentially like that described by Green and Stumpf.¹

It is to be noted in the accompanying table that regardless of the type of polysaccharide the activating effect is markedly increased by partial hydrolysis. Since corn-starch is about 80 per cent amylopectin, whereas the so called "butanol fraction" of corn-starch⁶ and synthetic starch are supposedly amylose polysaccharides,⁴ branching apparently may play some part in the activating power of a polysaccharide. Otherwise, the relatively small amount of amylose in corn-starch probably would not account for all the increase in activity observed.

* This work was supported by a grant from the Corn Industries Research Foundation to Indiana University.

¹ Green, D. E., and Stumpf, P. K., *J. Biol. Chem.*, **142**, 355 (1942).

² Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **135**, 733 (1940).

³ Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **151**, 57 (1943).

⁴ Hassid, W. Z., Cori, G. T., and McCready, R. M., *J. Biol. Chem.*, **148**, 89 (1943).

⁵ Hanes, C. S., *Proc. Roy. Soc. London, Series B*, **123**, 421 (1940).

⁶ Schoch, T. J., *J. Am. Chem. Soc.*, **64**, 2957 (1942).

Probably it is very significant that the amylopectin-rich polysaccharide reaches maximal activation sooner than the starches practically free from such branched chain material.

The staining reaction with iodine disappears completely about the time the polysaccharide begins to decline in activating power (see the table).

*Per Cent of Maximal Enzyme Activity**

	Length of hydrolysis, min.											Achro- mic point
	5	15	25	45	75	135	165	210	330	450	540	
Corn-starch.....	20.6	28.2	48.7	51.8	57.8	57.8	61.6	60.3	51.2	39.9		<i>min.</i> 240
Synthetic potato starch..	1.5		19.0	27.5	36.7	47.5	49.5	49.0	55.0	56.3	43.0	450
Schoch's "butanol frac- tion".....	28.2	31.9	35.6	40.4	45.7	57.4	60.1	55.8	65.0	65.4	67.0	555

* Maximal activity = activity in the presence of 20 mg. of soluble starch per determination.

These observations indicate that the activating effect in polysaccharide synthesis can be independent of linkages characteristic of amylopectin, although the latter may also have marked ability to activate the synthesis. Moreover, the behavior toward iodine suggests that extensive fragmentation of the polysaccharide molecules can occur without disappearance of activating ability. It does not seem probable that the effect is due to reversion products⁷ formed from glucose or other hydrolytic materials, because the activity declines after reaching a peak and eventually is abolished.

*College of Medicine
Baylor University
Houston*

PHIL HARTER HIDEY

*Department of Chemistry
Indiana University
Bloomington*

HARRY G. DAY

Received for publication, December 16, 1943

⁷ Myrbäck, K., *Svensk Kem. Tidskr.*, **53**, 67 (1941); *Chem. Abstr.*, **35**, 5098 (1941).

THE INFLUENCE OF DEXTRIN UPON THE SYNTHETIC ACTION OF PLANT PHOSPHORYLASE*

Sirs:

Through the work of Cori and Cori,¹ Hanes,² and others it has been found that liver phosphorylase acts upon Cori ester to form glycogen, that muscle phosphorylase *in vitro* forms material similar to starch, and that plant phosphorylase also forms a starch-like substance. It has been shown, furthermore, that the reaction proceeds very slowly unless a trace of glycogen or starch is present to act as a primer. In this laboratory we find that jack bean or potato phosphorylases can be primed with corn-starch amylose, corn-starch amylopectin, glycogen, erythrodextrin, achroodextrin, or either of the two water-soluble polysaccharides of sweet corn.³ We agree with Green and Stumpf⁴ and Green and Cori⁵ that pure maltose does not exert any priming action. Cori and Cori⁶ state that animal phosphorylase is not primed by amylose, which shows this enzyme to differ from plant phosphorylase.

We find that the nature of the product synthesized from Cori ester by plant phosphorylase depends upon the kind and amount of carbohydrate added to prime the reaction. Thus, a small quantity of achroodextrin will lead to the production of a substance giving a blue color with iodine. A larger quantity of achroodextrin will cause a product to be formed which gives a red color, while addition of a large amount of achroodextrin will cause the production of a substance that gives no color with iodine. In each case the quantity of inorganic phosphate liberated is practically the same. Our hypothesis is that the enzyme adds anhydro-*d*-glucose molecules to what foundation it finds present. If a few dextrin molecules are present, it forms chains sufficiently long to give a blue color with iodine. If many dextrin molecules are present, the phosphorylase forms many polysaccharide chains of intermediate length and the product resembles erythrodextrin. It is possible with phosphorylase preparations which contain amylase that the starch formed will be degraded to erythrodextrin

* With the financial assistance of the Rockefeller Foundation.

¹ Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **131**, 397 (1939); **135**, 733 (1940), especially p. 736.

² Hanes, C. S., *Proc. Roy. Soc. London, Series B*, **128**, 421 (1939-40); **129**, 174 (1940).

³ Sumner, J. B., and Somers, G. F., *Arch. Biochem.*, in press.

⁴ Green, D. E., and Stumpf, P. K., *J. Biol. Chem.*, **142**, 355 (1942).

⁵ Green, A. A., and Cori, G. T., *J. Biol. Chem.*, **151**, 21 (1943).

⁶ Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **151**, 57 (1943), especially p. 58.

as fast as it is formed. However, our potato phosphorylase has been found to be free from amylase.

Biochemistry Laboratory
Cornell University
Ithaca

JAMES B. SUMNER
G. FRED SOMERS
ELEANOR SISLER

Received for publication, December 22, 1943

THE PRODUCTION OF A HYDROXYPHENYL COMPOUND FROM *L*-PHENYLALANINE INCUBATED WITH LIVER SLICES

Sirs:

When *l*-phenylalanine is shaken with slices of rat or guinea pig liver in Ringer-bicarbonate solution, a product is formed which, after removal of protein, gives a positive test with Millon's reagent, diazotized *p*-nitroaniline, and α -nitrosonaphthol. It can be quantitatively estimated with the *p*-nitroaniline reagent, with tyrosine as a standard. Under optimal conditions, namely 300 mg. wet weight of tissue in 4.0 cc. of Ringer's solution containing 1.0 mg. of *l*-phenylalanine per cc., a yield of 10 to 15 per cent is obtained in 4 hours. Since the product is probably metabolized further (0.2 mg. of added tyrosine disappears under the same conditions), this yield probably represents the resultant of its rate of formation and disappearance. *d*-Phenylalanine produces less than half the amount of the chromogenic substance, and acetylphenylalanine, phenylethylamine, and phenylacetic acid produce none. Boiled liver slices, broken cell suspensions, slices under anaerobic conditions, and kidney slices are inactive. The Van Slyke estimation of the amino nitrogen gave the following results: added as *l*-phenylalanine, 0.339 mg. of $\text{NH}_2\text{-N}$; found after 4 hours incubation, 0.328 mg. This result was also obtained by Krebs.¹ The amino nitrogen of added tyrosine can also apparently be quantitatively recovered.

Continuous extraction with ether for 3 hours fails to remove the product from an acid or alkaline solution. Therefore no appreciable amounts of *p*-hydroxyphenylpyruvic or lactic acids are formed. Amyl alcohol fails to extract it from alkaline solutions, indicating that no tyramine is present. The product is also insoluble in benzene and xylene. Since it has been demonstrated² that tyrosine is formed in the rat from phenylalanine, it is possible that the product is tyrosine, although crystals have not yet been obtained.

*Departments of Biochemistry and Physiology and
Pharmacology
Duke University School of Medicine
Durham, North Carolina*

MARY L. C. BERNHEIM
FREDERICK BERNHEIM

Received for publication, January 3, 1944

¹ Krebs, H. A., *Z. physiol. Chem.*, **217**, 191 (1933).

² Moss, A. R., and Schoenheimer, R., *J. Biol. Chem.*, **135**, 415 (1940).

INFLUENCE OF CRYSTALLINE VITAMIN B₆ ON HEMATOPOIESIS IN THE CHICK

Sirs:

Following the isolation of crystalline vitamin B₆ in these laboratories by Piffner *et al.*,¹ we have made further studies as to its hematopoietic rôle in young chicks, determining the scope as well as the minimum and probable optimum protective levels.

The percentage composition of the synthetic vitamin B₆-free ration was as follows: purified casein 25, gelatin 10, *l*-cystine 0.3, choline chloride 0.2, corn-starch 52.4, cellulose 3.0, lard 4.0, salts² 5.0, and manganese

	Vitamin B ₆ per 100 gm. ration	No. of chicks	Body weight	Red blood cells		Hemo- globin	Leuco- cytes, per c.mm.	Thrombo- cytes, per c.mm.
					Hemato- crit			
	γ		gm.	millions per c.mm.	vol. per cent	gm. per cent		
Group 1. Basal syn- thetic B ₆ -free ra- tion	None	5	98	0.89	17.2	3.4	7,600	15,800
Group 2. Normal broiler ration		10	184	2.35	32.5	8.7	28,900	30,700
Group 3. Basal ra- tion with crystal- line vitamin B ₆	5	5	94	1.35	21.0	5.6	5,900	20,300
	20	4	129	1.92	31.5	5.9	18,200	31,900
	40	6	136	1.95	30.3	7.0	14,200	33,900
	100	7	185	2.01	30.1	7.6	23,500	38,600
	400	6	216	1.95	29.7	7.7	24,900	31,300

sulfate 0.1. It contained the following vitamins, per 100 gm.: vitamin A 1600 U.S.P. units, vitamin D 160 units, mixed tocopherol carrying 25 per cent α- 24 mg., vitamin K 5.0 γ, thiamine hydrochloride 0.4 mg., riboflavin 0.8 mg., pyridoxine hydrochloride 0.6 mg., *i*-inositol 50.0 mg., *p*-amino-benzoic acid 15.0 mg., niacin 2.0 mg., and sodium pantothenate 1.1 mg. In addition, crystalline biotin³ was given orally twice a week at a level equivalent to 1.5 γ per chick per day. The prophylactic test period was 4 weeks.

The data in the table for Groups 1 and 2, the basal synthetic and the normal broiler rations, show that the absence of vitamin B₆ brought about

¹ Piffner, J. J., Binkley, S. B., Bloom, E. S., Brown, R. A., Bird, O. D., Emmett, A. D., Hogan, A. G., and O'Dell, B. L., *Science*, **97**, 404 (1943).

² Jones, J. H., and Foster, C., *J. Nutr.*, **24**, 245 (1942).

³ We wish to thank Dr. R. T. Major of Merck and Company, Inc., for the generous supply of crystalline biotin.

a retardation in growth (body weight and feathering), a marked decrease in the hematocrit (Van Allen), the hemoglobin (Evelyn), and in the red blood cell count, and, in addition, a reduction in the leucocytes and thrombocytes. On the other hand, by fortifying the basal ration (Group 3) with crystalline vitamin B₁₂ in sufficient amount, this syndrome was prevented. Thus, for growth, the 20 γ level of the vitamin gave some protection but it required 100 γ to maintain the birds on the normal plane. In contrast with this, as little as 5 γ per 100 gm. of ration gave partial protection and 40 γ complete protection for the hematocrit, red blood cell count, hemoglobin, and thrombocytes. In the case of the leucocytes, the optimum requirement was very high, at about 400 γ .

In conclusion, under the conditions in which these experiments were done, the data show, for 4 week-old chicks, that vitamin B₁₂ is an essential factor for maintaining normal growth (and feathering) and for preventing macrocytic hypochromic anemia, leucopenia, and thrombopenia.

Research Laboratories
Parke, Davis and Company
Detroit

C. J. CAMPBELL
RAYMOND A. BROWN
A. D. EMMETT

Received for publication, January 17, 1944

IMPROVEMENTS IN THE TURBIDIMETRIC ASSAY FOR PENICILLIN

Sirs:

For some time we have had in use in this laboratory a modification of the Foster and Woodruff¹ method of assay of penicillin which has proved very useful. The importance and need of such a procedure prompt our describing it at this time. The method to be outlined is capable of giving results as quickly as that of Rake and Jones² and as accurate as the turbidimetric one of Foster and Woodruff¹ and Foster and Wilker.³ The shortcomings of the rapid method of Rake and Jones, such as the use of a pathogenic organism, the presence of many conditions subject to variation, and the somewhat subjective nature of the results, have been overcome. Strict aseptic conditions need not be observed in carrying out the test. The turbidimetric method of the above authors, while of comparable accuracy, requires 3 or 4 hours for completion of the test, whereas results may be had in 90 minutes with the present modification. The results are obtained in this short time by using as inocula a 2 to 3 hour culture of a rapidly growing streptococcus of Lancefield's Group B with incubation at 37° in a water bath. The strain of streptococcus used has been found to be sufficiently sensitive to penicillin.

The procedure which has been found to give satisfactory results is as follows: A solution of known penicillin titer and the unknowns are diluted with brain-heart broth to contain approximately 1 unit per ml., and these in turn diluted 1:5, 1:10, 1:20, 1:40, 1:50, and 1:100. Each tube, containing 2.0 ml. of one of the above dilutions, together with controls containing no penicillin, is inoculated with 0.10 ml. of a 2 to 3 hour brain-heart broth culture of Group B streptococcus. The tubes are swirled once and allowed to incubate in a 37° water bath until sufficient turbidity has developed to permit accurate reading, usually for 90 minutes or less. Growth of the organism is stopped at this point by adding 1 drop of 35 per cent formalin to each tube. Turbidities are obtained by the use of a Coleman universal spectrophotometer with the wave-length dial set at 650 m μ . Densities are measured against brain-heart medium blanks. The instrument was modified to accept directly the small tubes (10 $\times$ 75 mm.) used in the tests. Results may be obtained graphically, with known penicillin solutions as a standard, or one may interpolate from the readings of the standard. It is usually sufficiently accurate to assume an inverse linear

¹ Foster, J. W., and Woodruff, H. B., *J. Bact.*, **46**, 187 (1943).

² Rake, G., and Jones, H., *Proc. Soc. Exp. Biol. and Med.*, **54**, 189 (1943).

³ Foster, J. W., and Wilker, B. L., *J. Bact.*, **46**, 377 (1943).

relationship between the optical density (as a measure of growth) and the penicillin concentration over the short range which is indicated in the series of known penicillin concentration. One then obtains the true penicillin titer of the original solution, conveniently in Florey units per ml., on multiplying the determined activity by the dilution factor of the particular tube. When it is possible to estimate the original titer of an unknown with a degree of accuracy, two or three points of the above dilution series will fall in the usable range of the known, and thus checks may be obtained.

Wallace Laboratories, Inc.
New Brunswick
New Jersey

S. W. LEE
E. J. FOLEY
JEANNE A. EPSTEIN
J. H. WALLACE, JR.

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THE ELEVATION OF UTERINE β -GLUCURONIDASE ACTIVITY BY ESTROGENIC HORMONES*

Sirs:

β -Glucuronidase is a carbohydrase whose preparation, purification, and properties,¹ have been described only recently. In 1940,² indirect evidence was obtained for its probable rôle *in vivo*, that of catalyzing the synthesis of conjugated glucuronides. At that time, it was observed that the β -glucuronidase activity of liver, kidney, and spleen, and *not* of testis, ovary, uterus, and vagina, increased in animals fed glucuronidogenic substances which were non-estrogenic. It was then suggested that "the enzyme in these sex organs is not concerned primarily in the general detoxication processes of the body, but has some function connected with the metabolism or transport of the sex hormones." In this note we wish to report experiments in which the β -glucuronidase of various tissues of the mouse was determined after the administration of several of the "natural" estrogenic hormones. In addition, some non-estrogenic glucuronidogenic steroids and terpenes were tested as control substances.

Female mice weighing about 20 gm. were ovariectomized, and 21 days later the test substance was injected subcutaneously in a volume of 0.2 ml. of 8 per cent alcohol, twice a day for 3 days. A control group of ovariectomized mice received 8 per cent alcohol alone. All the animals were killed on the 26th day after ovariectomy, when the β -glucuronidase activity of various tissues of each animal was determined, as previously described.² The values obtained for uterus, and in some cases, liver, are compared here with values for the same organs of normal mice (see the table).

The findings may be pointed out: First, the level of the uterine β -glucuronidase in the control group of thirty-nine ovariectomized animals receiving 8 per cent alcohol alone corresponds to that of the enzyme in the 60 ovariectomized animals receiving non-estrogenic substances. This level is substantially lower than that found in normal mice. In the liver, no change is apparent in the enzyme concentration after ovariectomy. Secondly, in the forty-four ovariectomized mice which received estrogenic

* This study was aided by grants from the Medical Research Council and the Earl of Moray Research Fund, United Kingdom. The interest and helpful criticism of Professor G. F. Marrian are gratefully acknowledged.

¹ Masamune, H., *J. Biochem.*, Japan, **19**, 353 (1934). Oshima, G., *J. Biochem.*, Japan, **20**, 361 (1934); **23**, 305 (1936). Fishman, W. H., *J. Biol. Chem.*, **127**, 367 (1939); **131**, 225 (1939).

² Fishman, W. H., *J. Biol. Chem.*, **136**, 229 (1940).

substances, the uterine enzyme activity was elevated 100 per cent or more above the level in the control group. The liver enzyme concentration which was determined in the estriol-injected group remained essentially normal.

Condition of mouse	Substance administered	Amount injected	β -Glucuronidase activity*			
			No. of mice	Liver	No. of mice	Uterus
		γ		mg.		mg.
Normal	None		32	106.3 $\pm$ 21.1	19	94.4 $\pm$ 32.0
Ovariectomized	"		14	102.0 $\pm$ 16.0	39	47.8 $\pm$ 16.2

Estrogens

Ovariectomized	Estriol	72.0	4	106.5 $\pm$ 6.3	4	105.3 $\pm$ 24.3
"	"	7.2			11	137.1 $\pm$ 32.0
"	Sodium estriol glucuronide	72.0			6	139.7 $\pm$ 24.3
"	Estrone	7.2			12	169.1 $\pm$ 27.5
"	Estradiol	7.2			11	129.7 $\pm$ 36.2

Non-estrogens

Ovariectomized	Progesterone	7.2			10	28.0 $\pm$ 9.9
"	Pregnanediol	14.4			12	50.0 $\pm$ 20.0
"	Sodium pregnanediol glucuronide	7.2			14	38.6 $\pm$ 10.4
"	Borneol	7.2			10	50.8 $\pm$ 11.7
"	Menthol	7.2			14	38.6 $\pm$ 10.4

* The activity per gm. of tissue is expressed as mg. of glucuronic acid liberated in 70 hours of hydrolysis at 37.5° from sodium menthol glucuronide.³

It is indeed probable that β -glucuronidase, at least in the case of mouse uterus, may be concerned with the metabolism of the estrogens. The significance of these observations is the subject of research which is now in progress.

Department of Medical Chemistry
University of Edinburgh
Edinburgh, Scotland

WILLIAM H. FISHMAN³
LILLIAN W. FISHMAN

Received for publication, January 14, 1944

³ Royal Society of Canada Fellow (1939-40). Present address, Department of Biochemistry, Bowman Gray School of Medicine, Winston-Salem, North Carolina.

URINARY 17-KETOSTEROIDS IN METABOLISM*

I. STANDARDIZED CHEMICAL ESTIMATION

By RAYMOND L. CAHEN† AND WILLIAM T. SALTER

(From the Laboratories of Pharmacology and Toxicology, Yale University School of Medicine, New Haven)

(Received for publication, November 10, 1943)

Investigations of androgen metabolism hitherto have been impeded by the lack of reliable chemical methods for the quantitative analysis of urinary 17-ketosteroids. The classical Zimmermann reagent (1, 2) has given various values in different laboratories (3-11). These discrepancies lie in two categories. First, the final color developed is exaggerated by extraneous pigments. Secondly, the genuine color due to 17-ketosteroids varies not only for a single ketosteroid when analyzed by different individuals, but also for equimolecular concentrations of related ketosteroids.

The problem recently became even more complicated when Pincus (12, 13) reported another set of values resulting from the important application of his new antimony trichloride reagent. It is the purpose of the present report to show that these discrepancies can largely be reconciled when an appropriate technique is used. This technique has been extended to separate total 17-ketosteroids into *cis* (β) and *trans* (α) derivatives.

EXPERIMENTAL

Three features have been emphasized. First, the results obtained by the Pincus and by the classical Zimmermann methods have been compared. Secondly, the conditions of the Zimmermann procedure have been standardized so that a given concentration of any of the chief 17-ketosteroids would always develop the same degree of color, which should remain stable at a plateau level. Actually, the means was found of forming equal degrees of color from several ketosteroids when measured in equimolecular concentrations. Thirdly, extraneous pigments and undesirable chromogens were largely eliminated.

Materials—Samples of crystalline androsterone, dehydroisoandrosterone,

* This work was supported by the Jane Coffin Childs Memorial Fund for Medical Research and The International Cancer Research Foundation. The spectrophotometer was purchased with the aid of the Ella Sachs Plotz Foundation and the Fluid Research Fund of Yale University School of Medicine.

† Under the auspices of The Rockefeller Foundation. Present address, The Maltbie Chemical Company, Newark, New Jersey.

hydroxyetioallocholanone, and testosterone¹ were used in their free form after being dissolved in 95 per cent alcohol. They were kept in the ice box.

24 hour samples of urine were collected from healthy young girls (3, 5, and 6½ years old respectively). Toluene was used as preservative and the samples were stored in the ice box. In some cases samples of these urines were reinforced by the addition of androsterone and dehydroisoandrosterone.

Equipment—1 liter Pyrex flasks with interchangeable ground connections. 1 liter Pyrex separatory funnels. A constant temperature water bath automatically maintained at $25^{\circ} \pm 0.1^{\circ}$ for temperature control during the Zimmermann tests. Pyrex 10 cc. test-tubes calibrated at 2 cc. Micro burettes, 2 cc. in content, calibrated to 0.001 cc. and permitting the delivery of 0.001 cc. volumes. A water bath electrically heated to $80^{\circ} \pm 1.0^{\circ}$ for temperature control during the Pincus tests.

For careful qualitative determination of maximum absorption the Beckman spectrophotometer was used. The intensity of the colors developed routinely was measured by the Klett-Summerson photoelectric colorimeter.

Reagents—

Recrystallized pure *m*-dinitrobenzene in 2 per cent alcoholic solution, purified according to Callow and Callow (14).

2.00 N aqueous KOH (Eimer and Amend) solution.

5.00 N aqueous KOH (Eimer and Amend) solution.

Peroxide-free ether.

Aldehyde-free alcohol, 95 per cent and 60 per cent, purified by fractional distillation after boiling with KOH.

Chloroform, c.p. reagent.

Antimony trichloride, c.p. reagent.

Acetic acid, 95 per cent solution.

Procedure

The assay consists of four steps: (a) hydrolysis of the conjugated steroids; (b) extraction and separation of androgenic 17-ketosteroids; (c) development of the Pincus and Zimmermann reactions for the total 17-ketosteroids; (d) development of the Pincus and Zimmermann reactions for *cis* (β -) and *trans* (α -) 17-ketosteroids.

The technique used in our assays was as follows:

Hydrolysis of Conjugated Urinary Steroids—Before the extraction of the steroids hydrolysis was carried out according to the method described by

¹ For the samples of pure steroids used, we are indebted to Dr. E. Oppenheimer of the Ciba Pharmaceutical Products, Inc., Dr. Bernard J. Brent of Roche-Organon, Professor Louis Fieser, Dr. Seymour Lieberman, Dr. Konrad Dobriner, and Dr. I. T. Nathanson.

Holtorff and Koch (6). Determinations on urine, studied in duplicate for comparison of the methods of Holtorff and Koch (10 minutes boiling with 15 per cent HCl) and Consolazio and Talbott (15) (simultaneous extraction and hydrolysis), failed to show any consistent difference in the recovery of 17-ketosteroids. Moreover, the hydrolysis prior to the extraction is preferred for general use because it avoids special precautions against an inflammable solvent. An aliquot volume (250 cc.) of each 24 hour urinary sample was refluxed with 25 cc. of 12 N hydrochloric acid for 10 minutes. Then the mixture was cooled immediately in order to prevent the destruction of the free steroids which are thermolabile.

Extraction and Isolation of Free Urinary Steroids—For the extraction benzene and ether are more convenient as solvents than carbon tetrachloride (9, 15) because they extract completely both 17-ketosteroids and estrogens. In previous investigations we used benzene but we prefer ether which constitutes a less definite hazard. For the technique of extraction shaking in separatory funnels was used. Each extraction is practically complete after three shakings with 80 cc. of ether.

Following the extractions the neutral 17-ketosteroids were separated from the acid and phenolic fractions. After shaking first with 80 cc. of a saturated solution of sodium bicarbonate, then twice with 80 cc. of 15 per cent potassium hydroxide, and washing once with 80 cc. of water, the neutral ethereal fraction was left.

Decolorization with activated carbon (norit A, Merck) was performed in a few trial assays but was abandoned because of loss of active material, even when contact was limited to so short a time as 60 seconds.

The neutral ethereal fraction was then evaporated to dryness, first on the steam bath *in vacuo* and then in a desiccator. Thereupon it was transferred quantitatively with 96 per cent alcohol to volumetric flasks and later to 20 cc. storage bottles in which it was kept cold for further colorimetric assays.

Colorimetric Assay—The colorimetric determination of 17-ketosteroids was made on aliquot samples by either the Pincus or the Zimmermann reaction.

Pincus Method—The technique recently described was used (11) except for the modification of an incubation time of 60 minutes instead of 45 minutes, and the maintenance of the reaction mixture in the dark at a temperature of 20°. Prior to this incubation with acetic acid in the dark, the solution is heated with antimony trichloride for 20 minutes on a boiling water bath.

Zimmermann Method—In a preliminary series of experiments the procedure of Callow *et al.* (4) was followed as modified by Friedgood and Whidden (16). In this method 2 N aqueous KOH is employed to develop the color.

A second series of assays was conducted by the Holtorff and Koch (6) method in which 5 N aqueous KOH is used. In a third series of assays, this procedure was modified by extracting the color in chloroform.

Determination of Cis (β -) and Trans (α -) 17-Ketosteroids—*Cis* (β -) and *trans* (α -) urinary 17-ketosteroids were separated according to the method of Baumann and Metzger (17), which closely approximates the actual

TABLE I

Comparison of Zimmermann, Modified m-Dinitrobenzene, and Pincus Techniques for Urinary 17-Ketosteroids in Mg. per 24 Hour Secretion with Recovery of Added Ketosteroids

Age of girls	17-Ketosteroids added	Total 17-ketosteroids			Trans (α -) 17-ketosteroids			Cis (β -) 17-ketosteroids		
		Zimmermann	m-Dinitrobenzene	Pincus	Zimmermann	m-Dinitrobenzene	Pincus	Zimmermann	m-Dinitrobenzene	Pincus
	mg.									
K., 3 yrs.		3.8		3.3	3.3		1.8	0.25	0.25	0.25
		3.5	2.4	2.8	2.9	2.3	2.5	0.21		0.15
		2.9	2.0	2.3	2.4	1.9	2.1			
		3.5	2.5	2.3	3.3	2.0	2.0	0.23	0.18	0.20
Androsterone	12	15	14	14	14	13	13	0.23	0.18	0.21
Recovery, %		96	94	97	92	93	93			
Dehydroisoandrosterone	20	23	22	22	3.3	2.0	2.0	20	20	20
Recovery, %		97	98	98				98	99	98
C., 5 yrs.		4	2.8	2.4	3.6	2.0	2.0		0.24	
Androsterone	12	15	14	14	15	13	14		0.24	
Recovery, %		99	94	97	96	94	97			
Dehydroisoandrosterone	20	23	22	22	3.6	2.0	2.0			20
Recovery, %		95	96	98						90
F., 6½ yrs.		2.6	2.1	2.1	2.3	1.8	1.8			
		2.8	2.1	2.0	2.6	1.8	1.6	0.20	0.20	0.20
Androsterone	12	14	13	14	14	12	13	0.20	0.20	0.20
Recovery, %		96	93	98	94	87	92			
Dehydroisoandrosterone	20	22	21	21	2.6	1.8	1.6	20	20	
Recovery, %		96	95	95				98	98	

isolation values. For the final determination, an aliquot part of the alcoholic solution of urinary steroids was used and the respective *cis* (β) and *trans* (α) fractions were titrated colorimetrically by the Pincus or Zimmermann method as described above.

Results

The experimental data for the four steroids, each as determined by three methods, are presented in Figs. 1 to 3. In Table I are given the values

obtained from the children's urine, with and without addition of pure 17-ketosteroids.

Pincus Reaction—In the first series of experiments the absorption density of light, measured with the Klett-Summerson photocolormeter (Corning Filter 62), is compared for the three 17-ketosteroids after treatment with the Pincus reagent. Testosterone was similarly analyzed for comparison. In Fig. 1, the amount of steroid represented in 2 cc. of final solution is plotted against the direct logarithmic scale of the Klett-Summerson colorimeter (*i.e.*, the relative absorption). It will be noted that the curves for androsterone and hydroxyetioallocholanone are approximately linear.

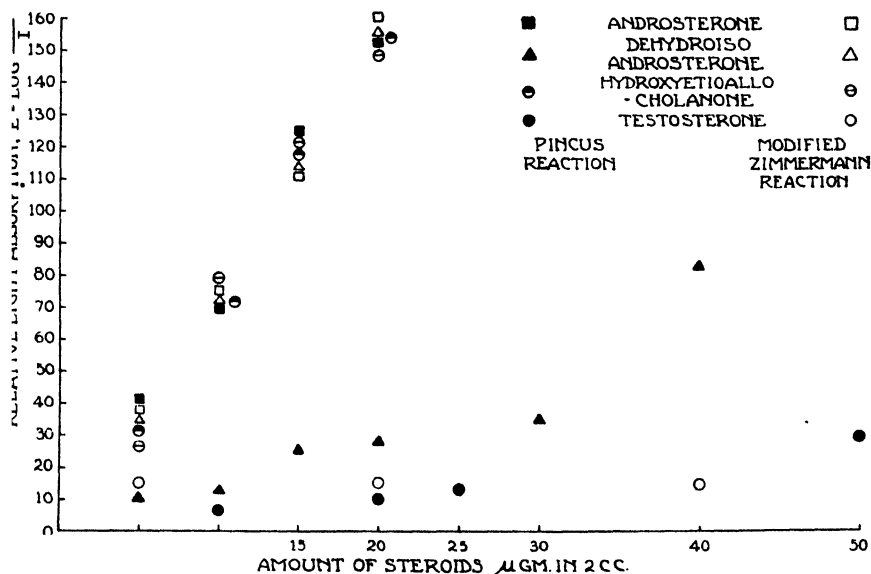


Fig. 1. Development of color by equivalent concentrations of several steroids after treatment (a) by the antimony trichloride reaction (Corning Filter 62) and (b) by the modified *m*-dinitrobenzene reaction (Corning Filter 52).

The curve for dehydroisoandrosterone is much less steep, and that for testosterone is close to the base-line.

Zimmermann Reaction—The next series of experiments concerns the influence upon reaction velocity of the concentration of the potassium hydroxide used in the original Zimmermann procedure (1). Two concentrations of KOH reagent are described; *i.e.*, 5.0 N and 2.0 N. The former concentration is used in the method of Holtorff and Koch (6), and the latter in the method of Callow *et al.* (4) as modified by Friedgood and Whidden (16). From Fig. 2 it is clear, first, that greater speed of reaction

is attained with the 5.0 N reagent, and, secondly, that after 105 minutes at 25° a plateau is reached. On the contrary with the 2 N reagent, the color does not reach a maximum even after a day's time, when the color becomes qualitatively distinctly yellow.

For these two reasons the 5.0 N alkali is preferred. The plateau found after 105 minutes confirms the result previously announced by Nathanson (18, 19).

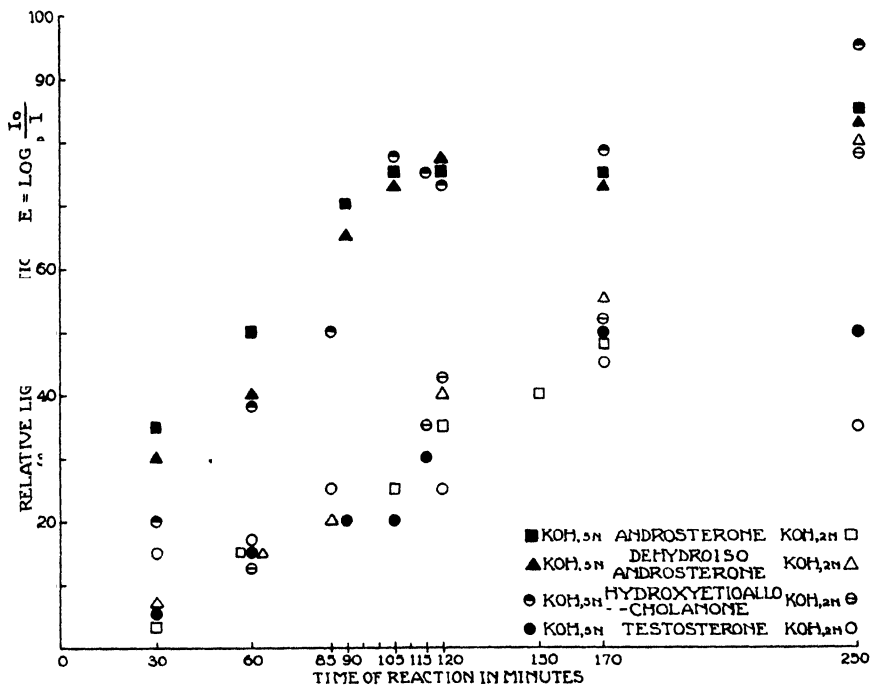


FIG. 2. Original Zimmermann technique (2). Effect of alkali concentration (5 N and 2 N) on the progressive development of color. A plateau is reached after 105 minutes.

Extraction of Color with Chloroform—Because it was observed that the blank develops considerable color on standing a few hours, an attempt was made at the end of the reaction to remove the excess of reagent or its breakdown products by extraction with a heavy organic solvent. Chloroform appeared preferable to carbon tetrachloride, carbon disulfide, and amyl alcohol. When 0.5 volume of chloroform was added to the alcoholic mixtures resulting from the Zimmermann reaction, and shaken as described below, this solvent extracted the desirable pink color and left the supernatant water-alcohol solution deeply tinged with yellow. For quantitative reliability, the following precautions had to be observed.

The mixture was shaken for ten oscillations, repeated twice at 3 minute intervals in the graduated 10 cc. test-tube. After each shaking enough chloroform was added from a micro burette to maintain the underlying chloroform moiety at 2 cc. volume. Because pure alcohol is miscible with chloroform, 60 per cent alcohol was necessary to prevent solution of the chloroform layer in the alcohol. The pink color is very photosensitive, but, as shown in Table II, it remained stable for 20 minutes in the dark, after which time it faded gradually. The colorimetric determination was made routinely at 10 minutes. Frequently an emulsion was formed, but could be

TABLE II
Stability of Pink Color in Chloroform Solution

	Steroid concentration	Time							
		0 min.	10 min.	25 min.	45 min.	95 min.	165 min.	205 min.	
		Relative absorption* (Corning Filter 52)							
	γ per 2 cc.								
Androsterone	5	33	33	32	30	30	30	30	20
	10	75	72	75	70	67	65	65	
	15	95	90	90	88	86	85	87	
	20	120	110	102	100	93	95		
Dehydroisoandrosterone	5	36	35		33	35		32	60
	10	85	77	75					
	15	116	112	112	104	105	95	100	
	20	135		133	130	125			
Hydroxyetioallocholanone	5	26	25	25	20	20		20	50
	10	75	73	70	69	72		78	
	15	126	120	110	110	100	102	100	
Testosterone	5	15	15	15		12	12	15	10
	20	15	15	14	12	15	15	12	

* Defined as $E = \log I_0/I = kcd$.

dissipated by placing the tube in a water bath at 40° for 1 minute. The chloroform solution was then sufficiently clear to be examined in the colorimeter.

Absorption Spectra—With the Beckman spectrophotometer the absorption curves of the water-alcohol and chloroform pigments were determined. In this work the authors are indebted to Dr. K. S. Salomon for his technical advice. As shown in Fig. 3, these absorptions differ considerably: only the chloroform solution shows a maximum near 450 m μ . Although the respective curves for the three 17-ketosteroids studied differ somewhat, they all show a maximum absorption between 430 and 480 m μ . Testosterone shows only a slight maximum.

Application to Human Urine—When the Klett-Summerson colorimeter was used with the Corning Filter 52, to determine total *cis* (β -) and *trans* (α -) 17-ketosteroids in human urines by the modified Zimmermann reaction, the results were consistent with those found by the Pincus reagent, with Corning Filter 62. As shown in Table I, however, the values are distinctly lower than by the original *m*-dinitrobenzene procedure. In some instances, the corresponding *cis* (β) and *trans* (α) fractions are also given,

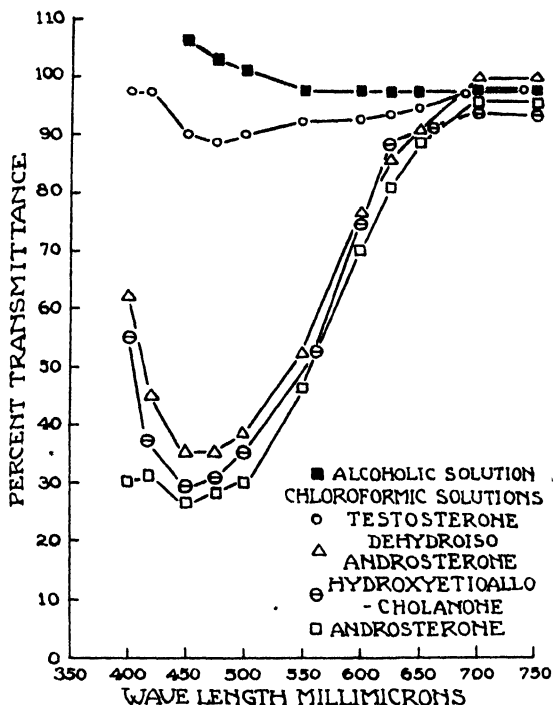


Fig. 3. Absorption curves of the colored compounds formed from several steroids by reaction with *m*-dinitrobenzene.

as determined separately. The sums of these are reasonably consistent with the total.

Table I shows also the recovery of known amounts of androsterone and of dehydroisoandrosterone when added to urine.

DISCUSSION

Most of the early results published for urinary 17-ketosteroids are too high, owing to the presence either of inactive chromogens or of preformed pigments (19). Callow and Callow (14) pointed out that normal urine

contains a non-specific fraction which gives a color value of 6 to 10 per cent of the total. Fraser *et al.* (20), Talbot *et al.* (21), and Pincus (22) confirmed these findings, although in a number of cases they obtained a much higher value for this fraction.

In order to determine whether a urinary extract is contaminated with such compounds, Callow and Callow (14) used the ratio of the absorption coefficients found with green and violet filters respectively. Fraser *et al.* (20), after comparing the respective readings of the color with these two filters, proposed a partial correction by an equation which reduced the error due to the non-ketonic chromogens. Pincus (22) has pointed out, however, that the large variability in the "*EG:EB* ratio" (light extinction, green to blue) may render such a correction invalid on statistical grounds. Furthermore, various urinary chromogens complicate the Zimmermann color titer even after the removal of non-ketonic material.

From our experience it appears that urinary pigments and chromogens do not appear in constant proportions. Moreover, they may vary qualitatively, so that sometimes the impurities have an absorption maximum which does not correspond to the filters ordinarily used for the correction factors. Therefore, a theoretical and rigid algebraic equation (23) may not be applied in every practical case.

The introduction of the Girard reagent (21, 11) made it possible to separate chemically the ketonic from the non-ketonic compounds and to this extent it is very useful. Nevertheless, the Girard complex is partially hydrolyzed even under the best conditions recommended by Girard and a constant loss of 10 to 15 per cent of the 17-ketosteroids appears during this separation (24). On the other hand, in our experience the Girard complex is likely to adsorb pigments from the impurities and these are carried into the solution of the regenerated ketones. Therefore, the use of the Girard reagent, which leads to important results in a qualitative sense, seems not to be ideal for quantitative colorimetric assays.

The use of the Pincus reagent is advantageous because it yields specific values, inasmuch as the absorption maximum of the green compounds formed by it is different from that of the contaminating red and brown pigments. The method has the further advantage of avoiding a color correction. According to our data, which confirm the results of Pincus (12), the values given by the SbCl_3 reaction are always lower than those given by the old Zimmermann reaction, even after subtraction of the urinary pigments as measured in a blank.

Why did the original *m*-dinitrobenzene technique give too high values? Because the Pincus reagent reacts very weakly with dehydroisoandrosterone, one might suppose that the determination of total 17-ketosteroids by the Pincus method would give distinctly lower results. Dehydroisoandros-

terone, however, normally occurs in relatively small quantities. At best, therefore, this feature would account only for a 10 per cent difference, whereas the observed discrepancy is usually about 40 per cent. This computation indicates that extraneous pigments are the source of the high values originally encountered with the dinitrobenzene reagent.

Are the extraneous chromogens already present in the urine or do they arise during the Zimmermann reaction? Because naturally occurring urinary pigments and chromogens have been eliminated satisfactorily by the blank, the extraneous pigments probably are formed *de novo* during the Zimmermann reaction. In actuality, this latter source is very important because water-alcohol solutions of pure 17-ketosteroids yield both pink and yellow pigments which can be separated by extraction. The intensity of the pink color is proportional to the amount of ketosteroid present, whereas the yellow pigment is independent of it. Although preformed urinary pigments are largely extracted by the chloroform, the optical blank corrects for this error. It is still possible, although unlikely on metabolic grounds, that the reagent yields a pink color with certain natural substances which are not 17-ketosteroids. The present data do not exclude this possible source of error.

Testosterone yields almost no color with the *m*-dinitrobenzene reagent. The color reported in the original Zimmermann technique is quantitatively accounted for by the transformed reagent itself.

SUMMARY

1. The Zimmermann reaction for 17-ketosteroids has been modified by extracting the resulting color in a heavy organic solvent. The supernatant layer retains in solution the undesirable chromogens and pigment impurities.

2. The titer given by this modified reaction, after correction for residual urinary pigments by comparison with a blank, is lower than that given by the original Zimmermann reaction, but is very close to that given by the antimony trichloride reaction of Pincus.

3. The titer given by the Pincus reaction or by the modified Zimmermann reaction, when the latter is read against the appropriate blank, does not reflect the non-endocrine urinary pigments.

4. Under the original Zimmermann procedure, testosterone gave a troublesome color. With the modified technique, however, testosterone yields practically no color. This result agrees with that by the Pincus reagent.

5. With dehydroisoandrosterone antimony trichloride yields a feeble color, whereas the modified Zimmermann reaction gives the same intensity as with other 17-ketosteroids.

6. The *m*-dinitrobenzene technique has been adjusted so that equivalent concentrations of various 17-ketosteroids yield the same color intensity.

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THE HIGHER FATTY ALDEHYDES

II. BEHAVIOR OF THE ALDEHYDES AND THEIR DERIVATIVES IN THE FUCHSIN TEST*

By MARJORIE ANCHEL AND HEINRICH WAELSCH

(From the Departments of Neurology and Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

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Recently, the isolation of the higher fatty aldehydes from animal tissues as acid hydrazones and oximes was reported (1). A method for the quantitative determination of these aldehydes is necessary for the study of their metabolic function. Feulgen and collaborators utilized the color developed by the aldehydes with the fuchsin sulfite reagent for this purpose (2, 3).

In this paper a detailed study of this color reaction is presented.

The use of a stable and reproducible standard is particularly necessary in the case of the fuchsin test, since the reagent varies from batch to batch. The free synthetic aldehydes, palmitaldehyde and stearaldehyde, are not stable and cannot readily be obtained in entirely monomeric form. Feulgen first used the thiosemicarbazones of synthetic aldehydes as standards, but later abandoned them, since they did not split completely and, therefore, gave low values (2, 3). He finally used the freshly steam-distilled aldehydes as an arbitrary standard (3).

As Bersin and Feulgen have put forward evidence that the higher fatty aldehydes occurring in nature are bound in acetal linkage to glycerol (4), synthetic glycerol acetals of the higher fatty aldehydes would appear to be the ideal standard. Palmitaldehyde and stearaldehyde glyceryl acetals were prepared according to the method of Bersin and collaborators (5) and were found to be satisfactory in regard to stability. According to Bersin, the synthetic acetals give maximal color development after 10 hours at room temperature. In our hands, the maximum was reached only after 18 hours at 37° (Fig. 2). Our synthetic acetals also behaved differently than did the naturally occurring aldehyde complex. Extracts of muscle and various organs of rats, cattle, and guinea pigs gave color within a few minutes under the conditions of the test and developed their maximal color within about 15 hours and probably in less time, at 5–10°. Under these conditions, the synthetic acetals yielded no color whatsoever even when tissue extract or a phosphatide preparation was added. Although

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the synthetic acetals develop maximal color only at 37° within a period of 18 hours, they may be used as standards in the determination of naturally occurring aldehyde complex, with which maximal color development is independent of the temperature between 5-37°.

The method of determination was basically that of Feulgen and collaborators (3) modified in two major respects: The color test was carried out at 37°, and for the extraction of the colored compound purified capryl alcohol (6) was used instead of amyl alcohol; troublesome emulsions and high blank values were thus avoided. The synthetic acetals were used as absolute standards in a study of the behavior, with fuchsin reagent, of preparations of palmitaldehyde, steartaldehyde, lower aldehydes, and of several aldehyde derivatives.

The modified method and the new standard were applied to the determination of the higher fatty aldehydes in tissues.

EXPERIMENTAL

Preparation of Palmitaldehyde and Steartaldehyde—The aldehydes were prepared by oxidation of the corresponding commercially available alcohols (stenol and cetyl alcohol flakes, du Pont) by the method developed by Schwenk and Bloch,¹ and isolated from the reaction mixture by the use of *p*-carboxyphenylhydrazine.

24 gm. of cetyl alcohol flakes and 8 gm. of cupric oxide were heated over the free flame in a current of nitrogen until most of the oxide had turned red. The product was cooled, dissolved in hot 95 per cent ethanol, and filtered into a hot solution of 10 gm. of *p*-carboxyphenylhydrazine in 95 per cent ethanol. A total of 300 ml. of ethanol was used. The addition of acetic acid (1) to the reaction mixture was found to be unnecessary. The solution was refluxed for 2 hours, and then distributed between 500 ml. of ether and 1000 ml. of a 4 per cent solution of potassium carbonate. The hydrazone was precipitated by acidification of the carbonate solution, and filtered under nitrogen. The yield of crude hydrazone was about 20 per cent of the theoretical, based on the alcohol used. For analysis the hydrazone was recrystallized twice from 95 per cent ethanol and dried over P_2O_5 at 2 mm. and room temperature. The carboxyphenylhydrazone of palmitaldehyde melted at 101-102° with decomposition; found, C 74.09, H 10.00; calculated for $C_{25}H_{48}O_2N_2$ (374.55), C 73.75, H 10.23. The carboxyphenylhydrazone of steartaldehyde melted at 105° with decomposition; found, C 74.63, H 10.24, C 74.44, H 10.59; calculated for $C_{28}H_{42}O_2N_2$ (402.61), C 74.58, H 10.52.² Formaldehyde was as effective

¹ We are highly indebted to Dr. E. Schwenk and Dr. E. Bloch for making their unpublished method available to us.

² For the microanalysis we are indebted to Mr. W. Saschek.

as pyruvic acid in liberating the aldehydes from their hydrazones. The hydrazone (0.01 mole) was refluxed with 8 ml. of 37 per cent formaldehyde (0.1 mole) in 100 ml. of 95 per cent ethanol for 4 hours. The reaction mixture was distributed between 300 ml. of 4 per cent potassium carbonate and sufficient ether, as previously described (7). For purification, the aldehyde was distilled at 2 mm. Palmitaldehyde distilled at 120–140°, stearaldehyde at 141–155°. The yields of once distilled product were 77 to 93 per cent of the theoretical, based on the hydrazone used. Sometimes preparations of palmitaldehyde or stearaldehyde were obtained which, in regard to their low melting point, solubility, analytical composition, and color equivalent, corresponded to the monomeric aldehyde. Some preparations made under the same conditions showed a higher melting point and lower color equivalent, apparently corresponding to products of polymerization or condensation of the aldehydes.

Preparation of Aldehyde Derivatives—The carboxymethoximes were prepared according to Anchel and Schoenheimer (7). Palmitaldehyde carboxymethoxime in a yield of 60 per cent of the theoretical was obtained, based on monomeric aldehyde. After two recrystallizations from methanol, it melted at 68–69°; found, C 69.05, H 11.13; calculated for $C_{18}H_{35}O_3N$ (313.47), C 68.97, H 11.25. Stearaldehyde carboxymethoxime in a yield of 40 per cent of the theoretical was obtained, based on monomeric aldehyde. After two recrystallizations from methanol, it melted at 81–82°; found, C 70.32, H 11.62; calculated for $C_{20}H_{39}O_3N$ (341.52), C 70.33, H 11.51.

The thiosemicarbazones were prepared according to Feulgen and Behrens (8): palmitaldehyde thiosemicarbazone, m.p. 109°; found, C 65.10, H 11.20; calculated for $C_{17}H_{35}N_3S$ (313.4), C 65.09, H 11.26; stearaldehyde thiosemicarbazone, m.p. 112°; found, C 67.02, H 11.20; calculated for $C_{19}H_{39}N_3S$ (341.4), C 66.78, H 11.51.

The acetals were prepared according to Bersin and collaborators (5) with the aid of the apparatus described by Clarke and Davis (9) adapted for liquids lighter than water.³ It was found that the acetal could be more conveniently purified after recrystallization from methanol by distillation (150–170° at 2 mm.). For analysis the acetal was distilled in a micro sublimation apparatus (bath 110°, 2 mm.): palmitaldehyde glyceryl acetal, m.p. 48–49°; found, C 72.63, H 11.89; calculated for $C_{19}H_{38}O_3$ (314.49), C 72.56, H 12.18; stearaldehyde glyceryl acetal, m.p. 57°; found, C 73.27, H 12.64; calculated for $C_{21}H_{42}O_3$ (342.5), C 73.61, H 12.36; molecular weight (Rast, camphor) 353.

Determination of Aldehydes with Fuchsin Reagent—The fuchsin reagent

³ A mixture of equal parts of phosphoric acid anhydride and sand was used as drying agent.

was prepared according to Feulgen and Grünberg (3). The capryl alcohol was purified by the addition of sodium and subsequent distillation.

The standards and the samples to be tested were dissolved in glacial acetic acid in a concentration of about 0.1 mg. per ml. and kept at room temperature. Two or three dilutions of this solution with glacial acetic acid were made (usually 3:1, 1:1, and 1:3) and 1 ml. portions of the original and the diluted solutions were used for the test.

To the acetic acid solution in a glass-stoppered cylinder were added 3 drops of 6 per cent mercuric chloride solution, 10 ml. of the fuchsin reagent, and 1 ml. of 1 N HCl. The contents were well mixed by shaking and the tightly stoppered cylinders were placed in a water bath at 37° for 18 to 24 hours. (If the cylinders were not tightly stoppered, the escape of SO₂ resulted in irregular values.) After cooling, the colored compounds were extracted by shaking for half a minute with 10 ml. of capryl alcohol, and the alcoholic solutions were cleared by centrifuging, transferred to the standard test-tubes, and read in the photoelectric colorimeter with Corning Filters 440 and 348.

Acetals As Standards—The suitability of the acetals as standards was studied with the above procedure. Solutions of palmitaldehyde and stearylaldehyde glyceryl acetals in acetic acid, containing about 0.1 mg. per ml., served as stock solutions. When such a solution is allowed to stand at room temperature, even for a period of 3 weeks, the color density, as measured with the same batch of fuchsin reagent, does not change. A solution tested after 6 weeks showed a decrease in density of about 10 per cent. The values for different preparations of the acetal were identical. Palmitaldehyde and stearylaldehyde acetals also gave identical values, showing that the difference in chain length within this range did not influence the color development within the error of the method. With different batches of the fuchsin reagent, wide differences in results were obtained with the same acetal preparation (Fig. 1, Curves 1 and 2), but the ratio of the extinction coefficients of various derivatives remained the same.

The influence of temperature on the color development of the acetals was tested by carrying out the determination both at 7° and at 37°. At 7° the synthetic acetal developed no color even after 24 hours. At 37° the maximum was reached after 18 hours (Fig. 2).

Fuchsin Test with Aldehyde Derivatives—All tests were carried out at 37° for 24 hours. Equimolar concentrations of the carboxymethoximes gave the same color values as did the acetals, but the carboxyphenylhydrazones and the thiosemicarbazones yielded only 66 to 85 per cent of the color developed by the standard. The stability of the hydrazones in acetic acid was studied by keeping a solution of 403 mg. of the *p*-carboxyphenylhydrazone of stearylaldehyde at room temperature for 24 hours. Dis-

tribution between ether and 4 per cent potassium carbonate solution gave 135 mg. of hydrazone, recovered from the carbonate, and 158 mg. of ether-

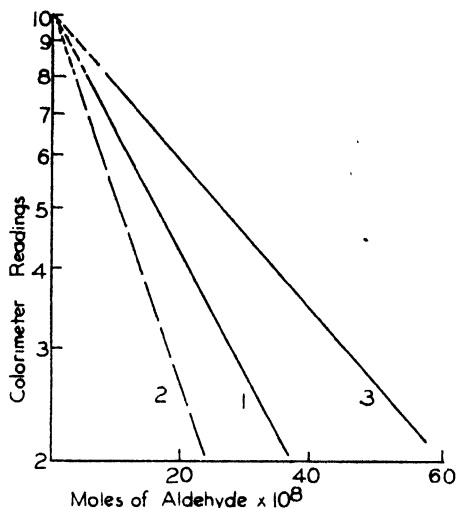


FIG. 1. Color values of acetals and hydrazones in the fuchsin test. Curves 1 and 2 represent palmitaldehyde glyceryl acetal and stearylaldehyde glyceryl acetal, with two different fuchsin reagents. Curve 3 represents the *p*-carboxyphenylhydrazones of palmitaldehyde and stearylaldehyde, with the same fuchsin reagent as in Curve 1. (This is almost 62 per cent color value.) Curve 1 was constructed from 67 points, of which 52 were within ± 5 per cent, the rest within ± 10 per cent. Curve 2 was constructed from twenty-four points, of which eighteen points were within ± 5 per cent, the rest being within ± 10 per cent.

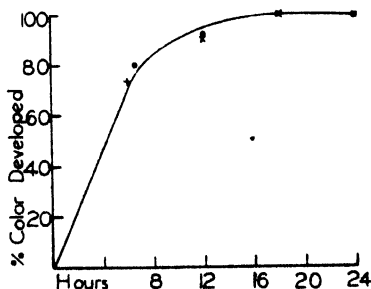


FIG. 2. Color development with palmitaldehyde and stearylaldehyde glyceryl acetals. ●, palmitaldehyde glyceryl acetal; X, stearylaldehyde glyceryl acetal.

soluble residue corresponding to 60 per cent of the aldehyde present in the original hydrazone.

Specificity of Fuchsin Reaction. Tests with Lower Aldehydes—The solu-

tions tested contained 1 mg. of aldehyde per ml. Formaldehyde (25 to 50 γ per ml.) gave a very deep color, of which only a very small portion was extracted by capryl alcohol, and which faded completely within 24 hours. Heptaldehyde, glyceraldehyde phosphoric acid, glyceraldehyde, and methylglyoxal yielded no color under the conditions of the test.⁴

Determination of Higher Fatty Aldehydes in Animal Tissue—The following experiments show the application of the method to the determination of the higher fatty aldehydes in the muscle, brain, and nerve of the rat and guinea pig when palmitaldehyde glyceryl acetal is used as standard. The values from one typical experiment with each of the tissues are reported. The determinations were always carried out in three different dilutions of the extracts, and in the case of muscle and brain, in duplicate. The values are expressed as mg. per cent of palmitaldehyde.

Muscle—Muscle tissue was minced finely with scissors on a watch-glass. A sample of about 1 gm. was suspended in 10 ml. of acetone (1), and shaken for an hour at room temperature. The liquid was decanted, and the extraction was repeated. The acetone extract was taken to dryness *in vacuo* and the residue was taken up in acetic acid (5 ml. per gm. of original tissue). The following values were found for abdominal muscle of a rat: 24, 24.6, 25, 25, 24, 28 mg. per cent.

Brain—Brain tissue was minced as described for muscle. A sample of about 100 mg. was extracted with 5 ml. of boiling 95 per cent ethanol. The mixture was centrifuged for a few seconds, the alcohol was decanted, and the extraction was repeated. The tissue was then extracted ten times with 2 ml. portions of boiling ether in the same way. The combined extracts were taken to dryness *in vacuo*, and the residue was taken up in acetic acid (10 ml. per 100 mg. of original tissue). The following values were obtained for the brain tissue of a rat: 327, 320, 340, 340, 340, 320 mg. per cent. Brain of guinea pig gave 310, 314, 280, (240), 310, 314, 300, 320 mg. per cent.

Nerve—A nerve sample of about 20 mg. was allowed to stand 1 hour in acetone, then removed and ground with sand. The mixture of tissue and sand was washed into a centrifuge tube with 5 ml. of 95 per cent ethanol and boiled with the alcohol for a few seconds. The mixture was centrifuged, and the extraction was repeated ten times with 2 ml. portions of boiling ether in the same way. The combined acetone and ether extracts were taken to dryness *in vacuo* and the residue was taken up in acetic acid

⁴ We are indebted to Dr. C. Neuberg for a sample of synthetic *dl*-glyceraldehyde and to Dr. O. Meyerhof for a sample of barium salt of triose phosphate containing 30 per cent of its phosphorus as phosphoglyceraldehyde. Owing to the insolubility of both preparations, saturated solutions were used which contained less than 1 mg. per ml.

(3 ml. per 20 mg. of original tissue). Two samples of rat nerve gave the following values: Nerve 1, 496, 556, 539 mg. per cent; Nerve 2, 510, 538, 478 mg. per cent.

Recovery of Synthetic Acetals from Tissue Extracts and from Mixtures with Egg Yolk Phosphatides—3 gm. of rat abdominal muscle were minced and divided into four parts. Two samples were extracted with acetone alone, two with acetone containing known amounts of acetal. For the preparation of the stock solution, the procedure described above was followed. The fuchsin test was carried out with two samples at 7°, and two samples at 37°, a control sample being paired at each temperature with one to which acetal had been added. The amounts of acetal added corresponded to 23 mg. of palmitaldehyde for 100 gm. of tissue.

The following values were found: at 7°, control 32 ± 3 mg. per cent, + acetal 32 ± 0 mg. per cent; at 37°, control 32 ± 3 mg. per cent, + acetal 42 ± 2 mg. per cent.

The recovery of acetal at 37° corresponded to less than half of the calculated value. It cannot be decided at this point whether this low recovery of the acetal is due to the loss of a part of the naturally occurring aldehydes in our procedure or to differences in the behavior of synthetic acetal and naturally occurring aldehyde complex.

Egg yolk phosphatides were prepared according to Feulgen and Grünberg (3). The fuchsin test was carried out with stearaldehyde glyceryl acetal with and without addition of 1 mg. of egg yolk phosphatide to each tube. In the ice box no color was developed in either case. At 37°, the acetals with and without addition of phosphatide developed maximal color.

DISCUSSION

The study of palmitaldehyde and stearaldehyde glyceryl acetals as standards in the determination of the higher fatty aldehydes has shown that these compounds are ideal from the point of view of convenience. They are stable in solution and give reproducible results. They can be handled at room temperature without liquefying, in contrast to the "monomeric aldehydes."

The color density obtained with the acetals is higher than that found with any of the other aldehyde derivatives studied, with the exception of the carboxymethoximes, which give the same values. This finding supports the assumption that the color yielded by these compounds corresponds to that given by the monomeric aldehyde. None of the preparations of "free aldehyde" ever produced a higher color density and the only samples of palmitaldehyde and stearaldehyde which gave values equal to

those of the acetal had the characteristics expected of the monomeric aldehydes.

The acetals develop full color with the fuchsin reagent within 18 hours only if the test is carried out at 37°. The free aldehydes develop color immediately and give the maximum in a shorter time at a lower temperature. The thiosemicarbazones and carboxyphenylhydrazones attain a maximal color corresponding to only 66 to 85 per cent of that of equimolar amounts of acetal. It appears that these compounds liberate the free aldehydes under conditions which favor the formation of non-chromogenic products; it is unlikely that the lower maxima are due to incomplete splitting, since with these compounds color development begins immediately. Moreover, it has been found that within 24 hours, the carboxyphenylhydrazone of stearylaldehyde decomposes to the extent of 60 per cent merely by standing in acetic acid solution at room temperature. This lability may explain why the *p*-carboxyphenylhydrazone is particularly suitable for aldehyde transfer (1).

The acetals were originally chosen as standards, not only on the basis of their stability, but also because, according to Feulgen and collaborators, the higher fatty aldehydes occurring in nature are bound in acetal linkage to glycerol. Therefore, it would be expected that the synthetic glyceryl acetals would most closely reproduce the behavior of the naturally occurring aldehyde complex toward the fuchsin reagent. But a comparison of the two showed that this was not the case. At the low temperatures at which the aldehyde complex from natural sources gave full color development, the synthetic acetal did not give any color. It may be reasoned that the presence of a phosphoric acid ethanolamine group in the plasmalogen would labilize the acetal linkage. But, according to Bersin and collaborators (5), both synthetic plasmalogenic acid and synthetic plasmalogen develop color more slowly than the simple glyceryl acetals. Our method of preparation differed from that of Bersin only in that the acetals were purified by distillation as well as by crystallization. From the elementary analysis and molecular weight determination it appears unlikely that the distillation could be responsible for an essential change in structure.

Bersin and Feulgen noticed that the isolated plasmalogen developed color with the fuchsin reagent at a slower rate than the compound present in tissue extracts. They attributed this behavior to the absence of other phosphatides serving as emulsifiers. The use of egg yolk phosphatides was recommended as an emulsifying agent in carrying out the fuchsin test. In our experiments the addition of phosphatides had no effect. Our results suggest that the aldehyde linkage in the naturally occurring complex may be more labile than the acetal linkage with glycerol.

Aldehydes can be determined with good reproducibility by the fuchsin test in extracts of tissues, but it is impossible to decide at this point whether all of the aldehyde present is determined, since the nature of the naturally occurring aldehyde linkage is uncertain. From the results with some derivatives (thiosemicarbazones, carboxyphenylhydrazones), it appears that the maximal color development of compounds containing the aldehyde is not necessarily equivalent to that of the free monomeric aldehydes.

SUMMARY

A quantitative study of the fuchsin test with derivatives of higher fatty aldehydes and with the free aldehydes themselves is presented. Palmitaldehyde and stearaldehyde glyceryl acetals are shown to be satisfactory standards in regard to stability in solution, and reproducibility. The acetals and carboxymethoximes of palmitaldehyde and stearaldehyde give the same maximal color development, which apparently corresponds to that of equivalent amounts of monomeric aldehyde. Other derivatives and different preparations of the free aldehydes give lower color maxima.

The synthetic acetals of the higher fatty aldehydes do not develop any color at low temperatures, whereas the aldehyde complex from natural sources develops full color. With acetals as standards, the fuchsin test must be carried out at 37°, at which temperature the maximum is reached within 18 hours. This finding casts some doubt on the glyceryl acetal as the naturally occurring form of the aldehydes.

The behavior of several naturally occurring lower aldehydes in the fuchsin test is described.

The fuchsin test has been applied to the determination of aldehydes in tissue extracts.

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THE DETERMINATION OF DEHYDROASCORBIC ACID AND ASCORBIC ACID IN PLANT TISSUES BY THE 2,4-DINITROPHENYLHYDRAZINE METHOD

By JOSEPH H. ROE AND M. JANE OESTERLING

(From the Department of Biochemistry, School of Medicine, George Washington University, Washington)

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In the customary method for the determination of dehydroascorbic acid the amount of ascorbic acid is estimated by oxidation-reduction techniques before and after reduction with H_2S . King (1) has shown that certain ketones and aldehydes on treatment with H_2S form compounds that respond like ascorbic acid in oxidation-reduction procedures, leading to a considerable error.

In a method, developed in this laboratory, for the determination of total vitamin C (2) an acid extract of tissue is passed through norit; this oxidizes ascorbic acid to dehydroascorbic acid, the bis-2,4-dinitrophenylhydrazone of which is treated with sulfuric acid, and the resulting red color is measured photometrically. We have adapted these analytical principles to the determination of dehydroascorbic acid in the presence of ascorbic acid. The procedure is similar to the above except that the acid-extracting solution contains 1 per cent thiourea and the oxidation by norit is omitted. The purpose of the thiourea is to stabilize the ascorbic acid during the extraction and subsequent treatment. Pure ascorbic acid dissolved in a solution containing 1 per cent thiourea and 5 per cent metaphosphoric acid, in a concentration of 15 γ per cc., gives no color after 5 hours coupling treatment with 2,4-dinitrophenylhydrazine at 37°. The assumption that no dehydroascorbic acid is formed from the ascorbic acid in plant tissues under the specified conditions is supported by the finding of some very low values for dehydroascorbic acid (Table I).

EXPERIMENTAL

Plant tissues purchased in a city market were ground in 5 per cent metaphosphoric acid solution. The ascorbic acid was photometrically determined in this extract by means of indophenol buffered at pH 3.6, according to the suggestion of Bessey (3). To 1 cc. of metaphosphoric acid filtrate in a colorimeter tube in an Evelyn colorimeter were added 9 cc. of indophenol containing appropriate amounts of sodium acetate according to the technique of Evelyn, Malloy, and Rosen (4). This procedure gave the values shown in Column 1 of Table I. Another portion of the filtrate was

TABLE I

Comparative Analyses of Plant Tissues for Vitamin C

The values are expressed as mg. per 100 gm. or 100 cc. of material.

Tissue	Ascorbic acid	Ascorbic acid + dehydroascorbic acid	Dehydroascorbic acid	
	Indophenol photometric method	2,4-Dinitrophenylhydrazine method	By difference (2) - (1)	Direct 2,4-dinitrophenylhydrazine
	(1)	(2)	(3)	(4)
Apple.....	3.1	4.5	1.4	0.0
Rhubarb.....	1.6	5.0	3.4	0.4
Lemon juice.....	57.6	59.5	1.9	0.5
Potato.....	28.1	31.6	3.5	0.5
Turnip.....	27.2	30.0	2.8	0.7
Asparagus.....	19.7	20.8	1.1	1.0
Carrot.....	5.4	8.3	2.9	1.0
Peach.....	1.3	3.3	2.0	1.0
Plum.....	1.2	3.6	2.4	1.2
Pineapple.....	15.6	21.0	5.4	1.3
Green peas.....	17.5	19.6	2.1	1.3
Cucumber.....	2.2	5.5	3.3	1.4
Grapefruit juice.....	40.3	45.0	4.7	1.6
Spinach.....	54.0	54.0	0.0	1.8
String beans.....	16.0	16.4	0.4	1.9
Lima beans.....	39.6	43.5	3.9	2.0
Lime juice.....	31.0	37.5	6.5	2.0
Simlin.....	14.7	17.4	2.7	2.5
Orange juice.....	69.0	74.0	5.0	3.4
Potato, dehydrated.....	99.4	111.0	11.6	5.0
Green pepper.....	169.0	173.0	4.0	5.2
Cauliflower.....	75.5	72.5	-3.0	6.3
Yellow squash.....	16.0	26.0	10.0	7.5
Parsley.....	209.0	219.0	10.0	8.3
Orange peeling.....	266.0	282.0	16.0	11.3
Grapefruit peeling.....	196.0	235.0	39.0	14.0
Kale.....	234.0	238.7	4.7	17.5
* Broccoli.....	108.0	115.0	7.0	18.0
Cabbage, dehydrated.....	391.8	405.0	13.2	18.7
“ “.....	512.0	540.0	28.0	22.5
Lemon peeling.....	204.0	210.0	6.0	25.0
Orange marmalade.....	21.2	23.0	1.8	2.4
“ “.....	4.4	7.1	2.7	0.8
Apple butter.....	0.8	2.6	1.8	1.8

then diluted 2 to 5 times with metaphosphoric acid and glacial acetic acid to make a final solution containing 5 per cent metaphosphoric acid and 10 per cent acetic acid. This was passed through norit and the total dehy-

droascorbic acid determined as previously described (2). The results are shown in Column 2 of Table I. The values for dehydroascorbic acid (Column 3) are obtained by subtracting each value of Column 1 from the corresponding value in Column 2. Their validity obviously depends upon the precision and specificity of the two methods used. The data of Column 4, Table I, were obtained by our direct procedure which will be described in detail below.

Direct Method for Dehydroascorbic Acid (for Plant Tissues Only)—The tissue is ground under usually 50 and never less than 20 parts of a solution containing 5 per cent metaphosphoric acid and 1 per cent thiourea. After filtration 4 cc. of extract are placed in each of three matched photoelectric colorimeter tubes. One tube is kept for a blank. To each of the others is added 1 cc. of 2 per cent 2,4-dinitrophenylhydrazine in approximately 9 N sulfuric acid. These are held at 37° for 3 hours, then cooled, together with the blank, in ice water. To each of the three tubes, while in the ice water bath, 5 cc. of 85 per cent sulfuric acid are added from a burette, a drop at a time, during not less than 1 minute. (Sulfuric acid, not grease, should be used for lubricating the stop-cock of the burette.) Finally 1 cc. of the 2 per cent 2,4-dinitrophenylhydrazine solution is placed in the blank tube. The tubes are shaken thoroughly under the ice water, removed to a rack, wiped dry after 30 minutes, and read in a photoelectric colorimeter, with a 540 m μ filter.

Colorimeter readings should be taken 30 to 45 minutes after removal from the ice bath. In some plant extracts a slight yellowish coloration appears when the 85 per cent sulfuric acid is added. This is compensated by the blank, to which the colorimeter is adjusted before the unknown is read. As the center setting does not remain constant in all cases, a greater increase in color sometimes taking place in the blank tube than in the unknown, the colorimeter readings should be made as directed above.

Appropriate standards must be run along with the unknowns, or, preferably, a calibration curve and chart are made with standard solutions of dehydroascorbic acid in concentrations ranging from 0.25 to 15 γ per cc. To prepare the dehydroascorbic acid standard, a solution of 25 mg. of ascorbic in 25 cc. of 5 per cent metaphosphoric acid is treated with 1 or 2 drops of bromine, shaken until yellow, decanted from the excess of bromine into a large test-tube, and aerated until colorless. Standards of appropriate concentrations are made by diluting with 5 per cent metaphosphoric acid containing 1 per cent thiourea.

DISCUSSION

Table I is of interest with respect to the methods for the determination of ascorbic acid. When little dehydroascorbic acid was present in the tissue,

the results by the indophenol and the dinitrophenylhydrazine method are in good agreement. In other analyses, marked differences can be accounted for by the value obtained for dehydroascorbic acid by our direct method. The agreement between the data obtained by the widely different methods is evidence for the specificity of each, and suggests possibilities of obtaining more reliable and precise values for true ascorbic acid than can be secured by bioassay techniques.

Our studies indicate that the indophenol method, as modified by Bessey (3), is reliable for the estimation of ascorbic acid in most plant tissues, and

TABLE II

Recoveries of Dehydroascorbic Acid Added to Plant Tissues When Analyses Were Carried Out by Direct Dehydroascorbic Acid Method

The values are in terms of micrograms per 4 cc. of filtrates of 1:50 dilution.

Tissue	Amount in control filtrate	Amount added	Amount obtained after addition	Amount recovered	Per cent recovery
Lemon juice.....	1.8	34.2	38.4	36.6	107
Orange ".....	2.4	34.2	37.8	35.4	104
Cauliflower.....	3.3	34.2	39.4	36.1	105
Spinach.....	3.7	34.2	38.7	35.0	102
Kale.....	5.5	34.2	43.6	38.1	111
Apple.....	0.0	33.2	32.4	32.4	98
Peach.....	0.0	33.2	33.2	33.2	100
Pepper.....	6.1	37.2	47.0	40.9	110
String beans.....	1.3	37.2	39.4	38.1	102
Yellow squash.....	2.5	37.2	40.5	38.0	102
Simlin.....	3.5	37.2	41.4	37.9	102
Lemon peeling.....	17.2	29.7	50.0	32.8	110
Orange ".....	13.2	29.7	47.0	33.8	114
Lemon " *.....	3.3	25.8	30.7	27.4	106
Orange " *.....	4.6	25.8	31.3	26.7	103

* Filtrate of 1:200 dilution.

that the 2,4-dinitrophenylhydrazine method is satisfactory for the determination of both ascorbic acid and dehydroascorbic acid in the same material. The indophenol method is obviously inadequate when dehydroascorbic acid is present in appreciable amounts.

The recoveries obtained by our direct dehydroascorbic acid method, when dehydroascorbic acid was added to a group of plant tissues, are shown in Table II. The high recoveries in the first experiments with lemon and orange peeling (110 and 114 per cent) were due to the use of too low a dilution, 1:50. When these analyses were repeated with an extract diluted 1:200, the recoveries were satisfactory.

One problem in vitamin C analysis is the possible interference in chemical methods by the reductone or enediol type of compounds that are formed by heating carbohydrate-containing foods in the presence of acid or alkali. These products rapidly decolorize indophenol (5) and also interfere in the dinitrophenylhydrazine methods. In Table III are summarized the results obtained by boiling carbohydrates in slightly acid, approximately neutral, and alkaline solutions.

TABLE III

Apparent Vitamin C Produced by Boiling Carbohydrates in Slightly Acid, Approximately Neutral, and Alkaline Solutions

All solutions contained 1 gm. of carbohydrate per 100 cc.

Carbohydrate	Treatment	pH		Apparent ascorbic acid	Apparent ascorbic acid + dehydroascorbic acid	Apparent dehydroascorbic acid	
		Before boiling	After boiling	Indo-phenol method (1)	Dinitro-phenylhydrazine method (2)	By difference (2) - (1)	Direct dinitro-phenylhydrazine method
Glucose	Distilled H ₂ O solution boiled 4 hrs.	5.5	4.7	0.03	0.04	0.01	0.02
Fructose		5.9	4.4	0.00	0.08	0.08	0.04
Dextrin		3.7	3.7	0.00	0.04	0.04	0.003
Glucose	Phosphate-buffered solution boiled 4 hrs.	6.8	6.4	0.15	1.10	0.95	0.79
Fructose		6.8	6.6	0.14	1.19	1.05	0.79
Dextrin		6.8	6.6	0.01	0.20	0.19	0.21
Glucose	0.5% NaHCO ₃ solution boiled 30 min.	8.3	8.5	13.1	20.0	6.9	4.9
Dextrin		8.3	9.2	2.4	3.4	1.0	1.6
Glucose	0.5% NaHCO ₃ solution boiled 90 min.	8.3	8.4	23.4	20.6	-2.8	6.3
Dextrin		8.3	9.4	10.2	3.1	-7.1	2.8

and alkaline solutions. After 30 and 90 minutes boiling of alkaline solutions of glucose and dextrin, relatively large amounts of apparent vitamin C were obtained by each method, but when solutions of glucose, fructose, or dextrin were boiled for 4 hours at pH 5.5 to 3.7, only traces of substances that interfere in either of the analytical methods were produced; the same treatment of similar solutions buffered with phosphate at pH 6.8 also gave relatively low values.

It appears from the data of Table III that interference in analytical methods for vitamin C as a result of the cooking process is not serious when the heating is carried out, as is customary, in neutral or slightly acid solu-

tions. In view of these results it is suggested that the higher values for vitamin C sometimes observed with the indophenol method with previously heated plant tissues are probably explained by the inactivation of the ascorbic acid oxidase of the tissue, with resultant preservation of the reduced form of the vitamin.

Adaptation of Original 2,4-Dinitrophenylhydrazine Method for Blood and Urine to Analysis of Plant Tissues—The 2,4-dinitrophenylhydrazine method of determining vitamin C was originally applied to blood and urine (2). As some laboratories have now adapted this method to other substances, we wish to make certain recommendations in regard to its application to plant tissues. Except for the need for a calibration curve and the use of a solution containing 5 per cent metaphosphoric acid and 10 per cent acetic acid instead of 6 per cent trichloroacetic acid for extracting the tissue, the procedure is essentially the same. Adsorption of the vitamin on the norit is counteracted by the presence of acetic acid. Trichloroacetic acid has the same effect, but in increased concentration it may crystallize out when the sulfuric acid is added later in the process, and so the use of acetic acid is preferable. Since the concentration of acetic acid influences the rate of coupling, the same amount of acetic acid must be used in preparing the calibration curve as in carrying out the analyses.

1 gm. of norit to 50 cc. of filtrate is recommended, but highly pigmented plant filtrates may have to be treated with norit two or three times to obtain complete clarification. When 10 per cent acetic acid was present in the extraction fluid, no decrease in value resulted when 3 gm. of norit per 50 cc. were added, but losses of 3.8 per cent were observed when 6 gm. were used. Filtrates containing 15 per cent acetic acid did not show diminished values when 8 gm. of norit per 50 cc. were added.

The sensitivity of the 2,4-dinitrophenylhydrazine methods requires the use of relatively dilute extracts, an advantage from the standpoint of interference from other substances. As a rule in making extracts we take an amount of tissue expected to yield a filtrate containing 0.2 mg. of vitamin in 100 cc. of filtrate, but this amount should not exceed 2 gm. per 100 cc. unless extremely small quantities of vitamin C are involved.

SUMMARY

1. A method for the direct determination of dehydroascorbic acid in the presence of ascorbic acid in plant tissues has been developed.

2. The dehydroascorbic acid of a metaphosphoric acid extract, in which the ascorbic acid is stabilized with thiourea, is coupled with 2,4-dinitrophenylhydrazine and the resulting derivative is treated with sulfuric acid to produce a red color which is measured photometrically.

3. Certain modifications of the original 2,4-dinitrophenylhydrazine method for the determination of the total vitamin C in blood and urine have been made in order to adapt this technique to the analysis of plant tissues.

4. A comparison of results obtained with thirty-two plant tissues by the indophenol photometric and the 2,4-dinitrophenylhydrazine method has been made. Good agreement of results by the two methods was observed except when much dehydroascorbic acid was present.

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MICRO GLASS ELECTRODE TECHNIQUE FOR DETERMINATION OF HYDROGEN ION ACTIVITY OF BLOOD AND OTHER BIOLOGICAL FLUIDS*

By C. LLOYD CLAFF† AND ORVAR SWENSON‡

(From the *Laboratory for Surgical Research, Harvard Medical School, Boston*)

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The amount of biological fluid available for determinations of hydrogen ion concentration is often small; for example, in this laboratory it was difficult to carry on end studies on tumors and fractures because of the small amount of fluid available for determinations several days after the start of an experiment. A new technique was therefore devised to measure the hydrogen ion activity in quantities of fluid as small as 0.005 ml. With standard acetate reference buffer solution and other buffer solutions with a tolerance error of ± 0.02 consistently reproducible results were obtained within the tolerance of the buffer solution; i.e., ± 0.02 . An advantage of the glass electrode described herein is the fact that if a capillary glass electrode tube is broken or clogged it can be discarded and another one used at once, without interruption or delay of the experiment. This method of filling the capillary reduces errors which might arise by reason of gas exchange.

Apparatus

The apparatus consists of three parts: a calomel half-cell, a fan-shaped Pyrex glass funnel for the 0.1 N HCl and the Ag-AgCl electrode, and a Corning glass No. 015 capillary tube 75 mm. in length.¹ The calomel half-cell is fitted with a reservoir for saturated KCl (Fig. 1). The outlet is raised a few mm. from the bottom of the reservoir to form a trap to catch crystals formed in the saturated KCl by change of temperature. This prevents crystals from clogging the tube or being carried down the tube and settling in the bottom of the saturated KCl drop where the liquid junction is formed, thus causing errors in determinations. Two stop-cocks control the formation of this drop (Fig. 1). The flattened funnel for hold-

* Aided in part by a grant from the American Medical Association.

† Research Fellow in Surgery, Harvard Medical School.

‡ Assistant and Arthur Tracy Cabot Fellow in Surgery, Harvard Medical School.

¹ These may be drawn to any desired diameter and stored in 0.1 N HCl. Special extremely fine capillary tubes are available from the National Technical Laboratories (micro tube No. 186-M). Our capillaries were drawn by Mr. James Graham, University of Pennsylvania Medical School, Philadelphia, Pennsylvania.

ing the 0.1 N HCl and Ag-AgCl electrode is made of Pyrex glass. It is 50 mm. long and 30 mm. deep, open at the top, so the capillary can be

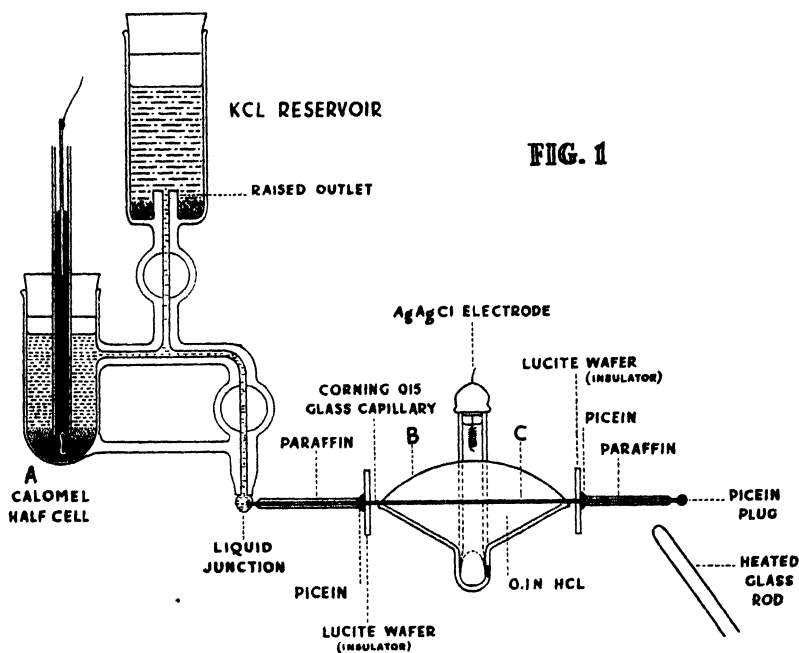


Fig. 1

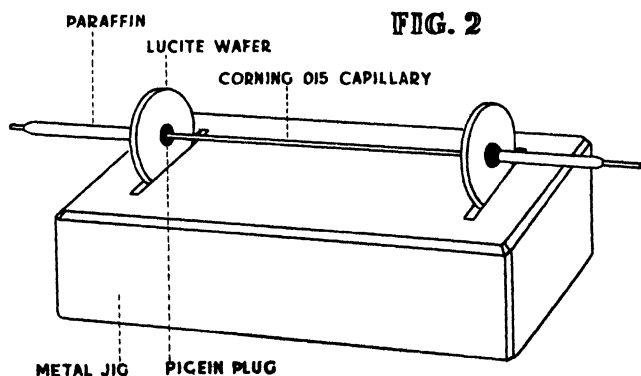


Fig. 2

supported across the opening. It is fitted with a side arm for holding the Ag-AgCl electrode. It has, also, a solid glass extension which enters a rubber holder. This in turn is fastened to a lucite arm capable of being raised and lowered by a rack and pinion movement as well as moving on an

end bearing in a horizontal plane. The 0.1 N HCl is introduced into the vessel with a pipette until it is filled to capacity. The surface tension holds the HCl from running out of the open ends.

The Corning No. 015 glass capillary is 75 mm. in length and has two lucite wafers attached to it by picein. The capillary assembly is made up in a jig (Fig. 2) so that the lucite wafers will always be the same distance apart. Starting 4 mm. from each end, the capillary is brushed with hot paraffin as far as the lucite wafers. This leaves the capillary between the wafers exposed (Fig. 1, *C*). The three elements are mounted on a lucite base and rod. The whole assembly is set up in an electrically shielded copper box connected by shielded cables to a No. 7660 Leeds and Northrup pH indicator.

Method

The capillary tube is cleaned thoroughly by sucking through it in the order mentioned Keego,² 0.1 N HCl, alcohol, distilled water, and 0.2 per cent potassium oxalate (to prevent blood clotting) by means of a small vacuum pump fitted to a water trap with pressure tubing. A small glass tube is fitted to the end of the pressure tubing and a short piece of pure gum rubber tubing is attached to it, which can be pinched over the end of the capillary while it is held. This makes a convenient way to apply suction to the end of the capillary tube. Next the capillary is thoroughly dried by sucking air through it. The fluid to be tested is taken into the capillary by a mouth pipette. The capillary is next placed across the open end of the funnel-shaped vessel (Fig. 1, *B*) with the lucite wafers resting just outside the rim of the vessel.

By means of the rack and pinion movement the whole assembly is raised into the hanging drop, thus making the liquid junction.

When micro capillaries (micro tube No. 186-M, National Technical Laboratories) are used, a slightly different technique is necessary. The fluid to be tested is taken up by capillary attraction. The capillary is filled to within 10 mm. of its end. When the tube is held with the unfilled part up, a plug of picein, heated over an alcohol lamp, seals the air chamber. The capillary is next placed across the open end of the funnel-shaped vessel (Fig. 1, *B*) as before.

The whole assembly is raised until the end of the capillary is just below the hanging drop. A heated glass rod is brought near the sealed end of the capillary to expand the air and push out the concave meniscus until a small bubble of liquid is formed on the end of the capillary. At this point the capillary is raised into the hanging drop, thus making the liquid junction.

² Wyandotte Keego cleaner, J. B. Ford Company, Wyandotte, Michigan.

If the above modification of technique is not followed, a small air bubble will be formed at the liquid junction due to the fact that the meniscus at the end of the small capillary is concave. This will invariably cause an error in the determination.

After the determination is made, the capillary assembly is removed (if it is the micro capillary, the picein plug is pulled off). The capillary is then thoroughly washed in Keego, 0.1 N HCl, alcohol, and distilled water, by sucking these through by means of the vacuum pump in the order named. It is then ready for a new determination, or for storage in 0.1 N HCl.

When capillaries are stored in 0.1 N HCl, the picein loosens around the capillary. When removed from storage, it is necessary, therefore, to dry the assembly with tissue paper, place in the jig, and reseal the lucite wafers to the capillary. Failure to do this will give erratic readings. The capillary assembly should be handled by holding the lucite wafer or the paraffined ends of the capillary, never the middle part between the wafers.

Fluid may be obtained from deep seated areas by inserting a No. 18 gage hollow needle into the site and allowing it to stand for a short period of time until enough fluid has collected in the lumen of the needle. The end of the glass capillary, thoroughly dried, can be inserted into the needle, filled by capillary attraction, and removed.

When not in use the fan-shaped glass holder should be drained and the Ag-AgCl electrode stored in 0.1 N HCl.

THE CHEMISTRY OF PHYTOMONAS TUMEFACIENS *

II. THE COMPOSITION OF THE ACETONE-SOLUBLE FAT

By SIDNEY F. VELICK AND R. J. ANDERSON

(From the Department of Chemistry, Yale University, New Haven)

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The crown-gall bacillus, *Phytoplasma tumefaciens*, stimulates in plants an abnormal cell growth which bears certain resemblances to malignant animal neoplasia (1). It has therefore been suggested that the action of the living bacillus is due to the liberation of growth-stimulating substances of a carcinogenic nature. Although it has not yet been possible to prove the presence of such a substance, it is of considerable interest that the lipids extracted from the bacterial cells have been shown to contain a fatty acid fraction (2, 3) which is similar in several respects to the branched chain fatty acids of the tubercle bacillus, one of which, phthioic acid, has marked cell-stimulating properties (4).

The present work is a continuation of the study of the lipids of *Phytoplasma tumefaciens*. The bacteria had been cultivated on a synthetic medium in which sucrose was the chief source of carbon. On such a medium the organism retains its virulence (5) and elaborates a phosphatide which contains liquid saturated fatty acids of high molecular weight as well as choline, ethanolamine, glycerophosphoric acid, and normal fatty acids (3).

The acetone-soluble fat, which is the subject of this report, was a reddish brown oil at room temperature. It contained about 70 per cent of free fatty acids. After saponification the fatty acids were separated by means of the lead salt-ether procedure into solid and liquid acids. The solid acids consisted mainly of palmitic acid. The liquid acids were subjected to catalytic reduction with hydrogen and platinum oxide, after which the lead salt-ether separation was repeated. The solid reduced acids contained a small amount of palmitic acid but the main component was stearic acid.

The liquid fatty acids, in addition to the unsaturated acids, contained a small amount of liquid saturated fatty acids. These acids formed lead salts that were easily soluble in ether and hence were contained in the ethereal extract obtained on treating the lead salts of the reduced acids with ether.

The water-soluble constituents isolated after the fat had been saponified formed a viscous syrup which gave no Molisch reaction but a positive

* This work was aided by a grant from The International Cancer Research Foundation.

acrolein reaction, thus indicating the presence of glycerol. It would appear therefore that the acetone-soluble fat was a mixture of free fatty acids and some neutral glycerides.

The liquid saturated fatty acids mentioned above were converted to the methyl ester and the latter was subjected to fractional distillation. A liquid saturated acid was obtained, the empirical formula of which appeared to be $C_{20}H_{40}O_2$. Owing to the small amount of material available, it was not possible to establish the purity of this acid conclusively. However, its properties were very similar to those of a branched acid of the composition $C_{20}H_{40}O_2$ isolated from the phosphatide in slightly larger amounts and subjected to a more rigorous purification (unpublished data).

As in the case of other pathogenic bacteria grown on a synthetic medium (6), no sterols could be detected in the unsaponifiable fraction. However, a peculiar new steam-volatile substance was isolated. It was purified by fractional distillation and found to consist largely of a compound of the formula $C_{12}H_{10}O$. Tests for aliphatic double bonds, hydroxyl, methoxyl, and carbonyl groups were negative. Both the composition and the physical constants corresponded with those of diphenyl ether.

Although the diphenyl ether structure forms part of the thyroxine molecule and is thus known to be synthesized in nature, it was considered desirable to repeat the isolation on new material. Through the courtesy of Dr. A. J. Riker an additional quantity of *Phytomonas tumefaciens* grown during the course of other investigations (7) was supplied. These bacteria differed from the ones previously extracted in that they had been lyophilized after isolation, whereas the previous material had been extracted in the moist state. There were also differences in the composition of the culture medium. On the basis of the former results the new material should have contained about 200 mg. of diphenyl ether. Actually only 25 mg. of a dark, strong smelling steam-volatile oil were obtained in which no diphenyl ether could be detected. It is possible that volatile material may have been lost during lyophilization or that because of the differences in the culture medium the bacillus, which has been shown to be sensitive metabolically to such changes (3, 8), did not produce diphenyl ether under the second set of conditions. Although the findings bear confirmation, the possibility cannot be excluded that the previously isolated diphenyl ether was a genuine metabolic product.

EXPERIMENTAL

The large scale cultivation of *Phytomonas tumefaciens* was carried out by Dr. A. J. Riker and associates in the Department of Plant Pathology at the University of Wisconsin. The synthetic medium contained 10 gm. of sucrose, 2.5 gm. of glutamic acid, 0.2 gm. of sodium chloride, 0.2 gm. of

magnesium sulfate heptahydrate, 0.1 gm. of calcium chloride, 1.0 gm. of dipotassium phosphate, and 7.5 gm. of ammonium sulfate per liter of water. After being tested for purity and virulence, the bacteria were isolated by centrifugation and frozen in the moist state in solid carbon dioxide, in which form they were shipped to New Haven.

*Extraction*¹—Immediately upon arrival each lot of bacteria was mixed with 1.5 volumes of alcohol and allowed to stand at room temperature under an atmosphere of carbon dioxide for 1 to 8 weeks. In all subsequent operations oxygen was excluded as much as possible by the use of carbon dioxide. After the bacteria had settled, the straw-colored fluorescent supernatant liquid was siphoned off and replaced with an equal volume of alcohol which was siphoned off after 7 days. The residues were now pooled and diluted with alcohol and ether until the total volume was 12 liters and the two solvents were present in equal amounts. The extraction was continued for 7 days with occasional shaking and was repeated twice. It was possible to separate the residues of the last two extractions by filtration. The bacteria were then extracted twice with 10 liters of chloroform and ether in a ratio of 1:1, and once with 10 liters of chloroform, each extraction as before being carried out for 7 days. The alcohol employed in the extractions was refluxed over potassium hydroxide and redistilled before use. The ether was treated with ferrous sulfate to remove peroxides and distilled over potassium hydroxide.

The alcohol and the alcohol-ether extracts were combined and concentrated to a small volume under reduced pressure, diluted with water, and extracted with ether. The chloroform extracts were treated similarly but were reextracted with chloroform. After the ether and chloroform solutions were filtered through a Chamberland filter, they were evaporated to dryness at 35°. Each fraction was dissolved in ether, filtered, and again evaporated to dryness. The ether-insoluble material was negligible. The total ether-soluble material weighed 79.81 gm. and corresponded to 6.4 per cent of the dry weight of the bacteria. The water-soluble portions, consisting chiefly of carbohydrate derived from the medium, were not further examined.

Acetone-Soluble Fat—Since all of the fractions were similar in appearance and solubility, they were combined and dissolved in 400 cc. of ether. To the resulting solution were added 800 cc. of acetone. The mixture was kept overnight in the ice chest and the supernatant liquid decanted from the gummy precipitate and combined with the subsequent acetone washings. The solvent was distilled off under reduced pressure and the residue was dissolved in acetone. A small amount failed to dissolve and was added

¹ The extractions were carried out by Dr. Alex Lesuk.

to the main acetone precipitate. Of the total lipids 41.7 per cent was soluble in acetone.

The acetone-soluble fat was a reddish brown oil which solidified in the ice chest and melted at 9°. A sample titrated in 90 per cent alcohol with alcoholic potassium hydroxide, a glass electrode being used, gave a rather flat curve with a slight drift near the inflection. The end-point indicated about 70 per cent free acid, calculated as oleic acid. The iodine number was determined by the Yasuda method (9) and found to be 71.9. There were 11.9 per cent of unsaponifiable material and a trace of steam-volatile acids (Reichert-Meißl number 2.7). The saponification number was 180.6.

For the isolation of the components the fat was saponified with alcoholic potassium hydroxide under an atmosphere of nitrogen on the steam bath for 3 hours. Without distilling off the alcohol, the solution was diluted with water and extracted with ether to remove the unsaponifiable material. The soap solution was then acidified and the acids were extracted with ether.

Water-Soluble Fraction—The aqueous residue after extraction of the acids gave a negative Molisch test and hence contained no carbohydrate. It was neutralized with sodium hydroxide and evaporated to dryness *in vacuo*. The residue was extracted with absolute alcohol, filtered, and again evaporated to dryness and extracted with alcohol. This process was repeated several times. A small amount of viscous dark liquid remained which gave a positive acrolein test for glycerol but did not yield a crystalline tribenzoate.

Unsaponifiable Fraction—During the determination of the Reichert-Meißl number small globules of a colorless oil collected on the aqueous distillate and sank to the bottom when the surface was disturbed. The distillate was neutralized and extracted with ether. After the extract had been washed with water, the ether was evaporated. The residue possessed a characteristic aromatic odor and was neutral in reaction. Accordingly the unsaponifiable fraction was subjected to steam distillation and more of the oil was obtained. In order to determine whether the material was chemically bound 6.62 gm. of the fat were steam-distilled without any previous saponification. The oil in the distillate weighed 0.40 gm. The residue was saponified and again steam-distilled, yielding an additional 0.14 gm. of oil. A total of 1.9 gm. of the oil was obtained from 27.9 gm. of the acetone-soluble fat. The non-volatile unsaponifiable material was a viscous reddish brown oil. It constituted 4.1 per cent of the acetone-soluble fat. The iodine number was 92.8. Tests for the presence of sterols were negative.

Purification and Identification of Diphenyl Ether—Elementary analysis of the crude steam-volatile material indicated the presence of oxygen. How-

ever, the material did not take up acetyl groups when refluxed with acetic anhydride and pyridine nor did it yield alkyl halide when refluxed with hydriodic acid in the micro-Zeisel apparatus. There was furthermore no detectable reaction with Schiff's reagent nor with dinitrophenylhydrazine. The oxygen thus appeared to be in a very stable linkage. It was eventually possible to purify the material by fractional distillation in an efficient small column at 20 mm. pressure. The middle fraction, which appeared homogeneous, crystallized in the receiver when supercooled and melted at 28°.

Analysis—8.844 mg. substance: 4.767 mg. H₂O and 27.34 mg. CO₂

C₁₂H₁₀O. Calculated, C 84.70, H 5.88; found, C 84.29, H 5.96

Found for diphenyl ether. n_D^{20} 1.5810, D_4^{20} 1.0728

“ “ 1.5809, “ 1.0719

p,p'-Dibromodiphenyl Ether—50 mg. of the purified material were dissolved in 0.5 cc. of carbon disulfide and 0.05 cc. of bromine was added. The solution was allowed to stand overnight, after which it was extracted with a few cc. of dilute sodium hydroxide, dried, and evaporated to dryness. The residue was recrystallized twice from methyl alcohol. It melted at 55.5–56° and gave no depression when mixed with a sample prepared from synthetic diphenyl ether (10).

Analysis—15.87 mg. substance: 25.45 mg. CO₂ and 3.76 mg. H₂O

C₁₂H₈OBr₂. Calculated, C 43.90, H 2.44; found, C 43.73, H 2.63

Examination of Second Batch of Phytomonas tumefaciens for Diphenyl Ether—The cells supplied by Dr. Riker for reexamination of the unsaponifiable fraction were originally grown in order to study the polysaccharide contained in the culture medium (7). The bacteria had been isolated by centrifugation and lyophilized. Of the dried bacterial cells 94 gm. were extracted in the manner previously described and the total ether-soluble portion saponified with alcoholic potassium hydroxide. The unsaponifiable material, isolated in the usual manner, weighed 331 mg. The material was suspended in water and steam-distilled until 600 cc. of distillate were obtained. The ethereal extract of the distillate yielded 25 mg. of a dark brown oil. It possessed a strong odor which was markedly different from the characteristic odor of diphenyl ether. The material was dissolved in carbon disulfide and half of the solution taken for preparation of the *p,p'*-dibromo derivative. The product obtained was a tarry substance insoluble in methyl alcohol and hence was not *p,p'*-dibromodiphenyl ether.

In order to make sure that no material had been missed 50 gm. of the extracted cell residues were hydrolyzed for 8 hours at 100° with 10 per cent sodium hydroxide. The hydrolysate was acidified and extracted three times with ether. No trace of steam-volatile material was found in the

ether extract. The non-volatile ether-soluble material upon evaporation weighed 300 mg. and consisted of solid fatty acids.

Solid Fatty Acids Isolated from Acetone-Soluble Fat—A sample of the fat weighing 14.90 gm. yielded on saponification 12.06 gm. of fatty acids, corresponding to 81.2 per cent of the fat. The acids were combined with material from the saponification of other samples and converted to the lead salts by precipitation of a hot aqueous solution of the potassium salts with neutral lead acetate. The lead salts were washed, dried *in vacuo* over calcium chloride, and extracted with ether. The insoluble salts were converted to the free acids by treatment with hydrochloric acid and ether. The solid fatty acids thus obtained constituted 8.1 per cent of the total acids. Two more lead salt fractionations of the solid acids removed an additional small quantity of liquid acids. The solid acids after this treatment absorbed no iodine.

The methyl esters of 0.93 gm. of the solid acids were prepared by treatment with diazomethane. After the esters were washed and dried, they were fractionated in a small Craig still (11). The pot temperature was raised 5° per hour and the column maintained 10° lower. Fractions 2 and 3 appeared to be fairly pure methyl palmitate, m.p. 29.2°, and on saponification yielded an acid which after two recrystallizations from methyl alcohol melted at 62.5°.

Analysis—9.52 mg. acid: 26.22 mg. CO₂ and 10.53 mg. H₂O

C₁₆H₃₂O₂. Calculated, C 75.0, H 12.5; found, C 75.1, H 12.3

Titration—54.50 mg. acid required 2.64 cc. 0.0807 N KOH; neutral equivalent 256, calculated for C₁₆H₃₂O₂ 256

Fractions 4 to 6 contained slightly increasing amounts of a higher homologue, probably stearic acid. However, the higher components were present in such small amounts that no pure acid could be isolated.

Liquid Acids—The liquid acids were reduced in alcoholic solution with hydrogen and platinum oxide. Although the major portion of the hydrogen absorption occurred in 3 hours, the solution was allowed to stand under slight hydrogen pressure overnight. After removal of the catalyst and distillation of the solvent at reduced pressure the product was saponified to remove any ester that had formed and was then subjected to a lead salt fractionation. From 18.11 gm. of liquid acids there were obtained 16.11 gm. of solid reduced acids.

5 gm. of the solid reduced acids were esterified with diazomethane and fractionated in a small, jacketed Widmer column at a pressure of 3 mm. In the first distillation three fractions were taken. Fraction 3 was almost pure methyl stearate. Redistillation of the other fractions yielded more methyl stearate, bringing the total up to 54 per cent of the solid reduced acids. The ester melted at 38.3°. It was saponified with alcoholic potas-

sium hydroxide and the free acid was crystallized from methyl alcohol. It melted at 69.6°.

Analysis—11.87 mg. acid: 33.04 mg. CO₂ and 13.46 mg. H₂O

C₁₈H₃₆O₂. Calculated, C 76.1, H 12.7; found, C 75.9, H 12.6

The intermediate fractions were then systematically redistilled, yielding an impure ester of palmitic acid which constituted about 10 per cent of the material and a series of fractions of continuously varying composition which appeared to contain no component other than palmitate and stearate. All of the distillation residues were combined and transferred to the Craig still and distilled until the total residue from the 5-gm. of starting material weighed less than 70 mg. No trace of any acid higher than stearic acid could be found. Since the amount of solid acids obtained after reduction was 93 per cent of the original liquid acid fraction, the unsaturated acids must have consisted mainly of oleic acid together with a small amount of palmitoleic acid.

Liquid Saturated Acids—The ether-soluble lead salt fraction of the reduced liquid acids was decomposed in the usual manner and the free acids isolated. Since the iodine number was found to be 2.5, the acids were reduced a second time and put through two more lead salt fractionations. The resulting material was a viscous brown liquid which weighed 1.05 gm., corresponding to 5.8 per cent of the total fatty acids. It was saturated, since it absorbed no iodine and it formed lead salts which were completely soluble in ether.

The methyl esters were prepared by the action of diazomethane and were distilled at 1 mm. pressure in a small Widmer column. Three fractions of approximately equal size were taken. There was a steady change in the index of refraction. Solidification occurred when the ester fractions were immersed in an ice-salt bath. When the material was allowed to come slowly to room temperature, it first became an opaque fluid, then gradually cleared. This behavior indicated the presence of a solid component in all of the fractions.

After several attempts at fractionation by other methods the fractions were combined, reesterified, and distilled in a specially constructed column which will be described in a subsequent report. Twelve fractions were taken from 580 mg. of methyl esters (Table I).

The fore run consisting of six small fractions contained at least three components in less than 250 mg. of material and was therefore not examined in detail. Fraction 6 upon saponification yielded an acid which after three recrystallizations from methanol and acetone weighed 20 mg. It melted at 63° and had a neutral equivalent of 288 and was hence chiefly stearic acid. Fractions 7 to 10 indicated the emergence of a pure com-

ponent. The index of refraction showed a plateau and the methyl esters remained clear liquids at 0°. Since the amount of material was not sufficient for redistillation, the combined fractions were analyzed.

Analysis—11.47 mg. methyl ester: 32.44 mg. CO₂ and 13.20 mg. H₂O
C₂₁H₄₂O₂. Calculated, C 77.50, H 12.88; found C 77.12, H 12.79

The carbon and hydrogen values correspond approximately to the calculated values of the methyl ester of an acid having the composition C₂₀H₄₀O₂. The remainder of the ester was saponified with alcoholic potassium hy-

TABLE I
Distillation of Methyl Esters of Liquid Saturated Acids from Acetone-Soluble Fat of Phylomonas tumefaciens

Fraction No.	Column temperature	Refractive index	M. p. of ester	Weight
	°C.	<i>n</i> _D ²⁵	°C.	gm.
1	146	1.4442	6	0.037
2	146	1.4445	10	0.030
3	146		10	0.026
4	149	1.4471	<0	0.040
5	151	1.4471	3	0.022
6	154		18–26	0.060
7	154	1.4490	6	0.109
8	154	1.4500	<0	0.062
9	154	1.4500	<0	0.065
10	160	1.4502	<0	0.033
11	165	1.4514	3	0.063
12		1.4517	5	0.027

droxide and the free acid isolated. It was liquid at room temperature but solidified in ice water and melted at about 15°.

Titration—24.26 mg. acid required 4.76 cc. 0.01654 N KOH; neutral equivalent 308, calculated for C₂₀H₄₀O₂ 312

SUMMARY

1. *Phytomonas tumefaciens* grown on a medium in which sucrose is the chief source of carbon contains 6.4 per cent of lipids of which 41.7 per cent is acetone-soluble fat.

2. The acetone-soluble fat contains approximately 70 per cent free acid.

3. The unsaponifiable matter contained a steam-volatile substance which was identified as diphenyl ether. This compound was not found in organisms grown under slightly different conditions.

4. The acetone-soluble fat contained a small amount of solid saturated fatty acids, mainly palmitic acid, and a large amount of unsaturated acids

which yield mainly stearic acid on reduction, but a small amount of palmitic acid was also present.

5. A liquid saturated fatty acid was isolated, of the probable empirical composition $C_{20}H_{40}O_2$.

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THE CHEMISTRY OF PHYTOMONAS TUMEFACIENS*

III. PHYTOMONIC ACID, A NEW BRANCHED CHAIN FATTY ACID

By SIDNEY F. VELICK

(From the Department of Chemistry, Yale University, New Haven)

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Anderson and coworkers have shown that both the acetone-soluble fat (1) and the phosphatide (2) of the crown-gall bacillus, *Phytomonas tumefaciens*, grown on a simple synthetic medium, contain fatty acids which differ markedly in physical properties from the members of the normal aliphatic series. Owing to the limited quantity of material, it was previously not possible to effect a complete purification of these acids. It was shown, however, that a major component of the so called liquid saturated acid fraction of the acetone-soluble fat contained an acid of the probable empirical formula $C_{20}H_{40}O_2$.

With the aid of an improved method for the fractional distillation of small amounts of material the same acid has now been isolated from the phosphatide in apparently pure form. Purification was effected by fractionation of the lead salts, followed by fractional distillation of the methyl esters. The methyl ester, a colorless mobile liquid at -15° , was found to have the composition $C_{21}H_{42}O_2$. It was converted in good yield to the crystalline hydrazide ($C_{20}H_{42}ON_2$) which melted at 56.6° . The free acid obtained by saponification of the methyl ester formed a solid crystalline mass. Crystalline fragments separated from the mass were in the form of thin transparent plates which melted at 24° and showed complete extinction when rotated between crossed nicol prisms. The neutral equivalent and carbon and hydrogen values corresponded closely to the formula $C_{20}H_{40}O_2$. The ester showed no detectable optical rotation in 8.1 per cent solution in ether.

The melting points of the acid and its derivatives are more than 50° lower than those of the corresponding straight chain compounds and 30 – 50° lower than those of mixtures of corresponding homologous normal compounds of comparable molecular weight. The acid absorbs no bromine and its lead salt is easily soluble in ether. Since the elementary analysis renders a cyclic structure unlikely, it would appear that the acid has a branched chain structure. Its properties suggest that it is a possible homologue of tuberculostearic acid, isolated from the tubercle bacillus (3) and for which the structure 10-methylstearic acid has been proposed (4).

* This work was aided by a grant from The International Cancer Research Foundation.

The separation of the branched chain acid fraction from the normal unsaturated acids required a catalytic hydrogenation of the latter. The molar ratio of double bonds in the original mixture to the normal saturated acids isolated after reduction was approximately unity. It is therefore concluded that the branched chain acids occurred in the bacterial cell in saturated form and were not reduced during the hydrogenation.

The crude branched chain acid fraction subjected to distillation weighed 2.3 gm., which was equivalent to 14 per cent of the total fatty acids. Of this material 0.889 gm. or 5.4 per cent of the total fatty acids was isolated as pure phytomonic acid by fractional distillation. An estimated additional 200 mg. were also distributed in intermediate fractions. The fore run which consisted of fourteen small fractions contained three or more components, including a small amount of stearic acid. There was also a small amount of higher boiling material which was not present in sufficient amounts for redistillation but appeared to contain branched components.

EXPERIMENTAL

The bacteria employed in this work were a controlled strain of *Phytomonas tumefaciens* cultivated on a simple synthetic medium in which sucrose was the chief source of carbon by Dr. A. J. Riker and coworkers in the Department of Plant Pathology at the University of Wisconsin. The extraction of the lipids from this material was described in the preceding report (1). After repeated precipitation from ether solution by the addition of acetone the phosphatides were obtained as a straw-colored granular powder which readily formed a colloidal solution in water. In confirmation of earlier work (2) the water-soluble components of the hydrolysate were found to be ethanolamine, choline, and glycerophosphoric acid. It was found in addition that upon treatment of an aliquot of the water-soluble portion of the hydrolysate with ninhydrin an amount of carbon dioxide equivalent to 15.5 per cent of the amino nitrogen was liberated, indicating the presence of an amino acid which could not be identified. The Molisch reaction for carbohydrate was negative. For analysis the material was dried *in vacuo* at 78° over phosphorus pentoxide.

Analysis—18.71 and 22.22 mg. phosphatide; 62.92 and 74.34 mg. ammonium phosphomolybdate; found, P 4.85, 4.83

21.39 mg. phosphatide (Kjeldahl); 3.44 cc. 0.00998 N HCl; found, N 2.24

Fatty Acid Fractions—The phosphatide was hydrolyzed by refluxing with 5 per cent sulfuric acid under nitrogen for 14 hours. When the hydrolysate had cooled, the acids were extracted with ether and the ether solution was washed with water and evaporated to dryness. The acids so obtained were saponified by refluxing with 10 per cent alcoholic potassium hydroxide for 2 hours. The soap solution was diluted with water

and extracted with ether in order to remove any unsaponifiable material. The potassium salts were converted to the free acids in the usual manner and the latter converted to the lead salts and extracted with ether. Less than 0.3 per cent (0.050 gm.) of the total fatty acids formed ether-insoluble lead salts.

The iodine number of the liquid acids, determined according to Yasuda (5), was 70, and the neutral equivalent 287. In order to remove the normal unsaturated acids the mixture was reduced with hydrogen and platinum oxide in alcoholic solution. The catalyst was filtered off and the solution was concentrated under reduced pressure and refluxed with potassium hydroxide for a short time to saponify any ester that had formed. The free acids were then isolated and separated into solid and liquid fractions by the lead salt-ether procedure. The solid acids after regeneration from the lead salts by treatment with hydrochloric acid and ether weighed 13.6 gm., which was 82 per cent of the original liquid acids. On distillation of the methyl esters they were found to consist of 11 per cent palmitic acid and 89 per cent stearic acid.

The ethereal extract obtained from the lead salts of the reduced acids as mentioned above yielded 3.1 gm. of acids which were liquid at room temperature and were fully saturated, since they absorbed no bromine. After two additional lead salt-ether fractionations no further solid impurities could be detected in the liquid acids by this method.

Isolation of Phytomonic Acid—The fractionating column constructed for the distillation of small amounts of high boiling material under reduced pressure consisted of a 30 cm. Pyrex tube of 4 mm. bore packed with a glass spiral and surrounded with a double air jacket electrically heated. The still head contained a capillary arm and a supplementary heater which permitted a continuous partial take-off under conditions of total condensation. An all-glass rotating receiver allowed the separation of samples as small as 20 mg. The resolving power under operating conditions was estimated by the distillation of standard mixtures and found to be eight to nine theoretical plates, which was sufficient for the separation of mixtures of methyl laurate, myristate, palmitate, and stearate. Details of construction, operation, and performance will be reported separately.

A sample of the liquid saturated fatty acids obtained from the ether-soluble lead salts was esterified with diazomethane and transferred to the distilling flask. The distillation of 2.3 gm. of material took 30 hours and covered a boiling range of 80° at a pressure of about 3 mm. The progress of the fractionation was followed by surrounding the receiver with an ice bath and observing the melting point of successive drops of distillate. The column temperature was raised whenever the distillation rate slowed down appreciably or stopped over the period of an hour. In Table I are the results of the fractionation. Because of the necessity of conserving material in the

very small fractions the index of refraction was omitted in most of the cases in which the melting point showed a progressive change. It should be noted that the jacket temperature does not represent the true boiling point.

It can be seen from the trend of the melting points and the rise in the index of refraction that the material in the fore run (Fractions 1 to 15) was a complex mixture containing at least two higher melting and one or two low melting esters. The emergence of the major liquid component was apparent in Fractions 15 and 16 which solidified in an ice-salt bath and, on slowly warming to room temperature, formed two component systems which gradually cleared between 4° and 10°. Fraction 17 developed only a turbidity at -15° and the next three fractions, which constituted 38.7 per cent of the material, remained a clear mobile liquid at -15°.

TABLE I
Distillation of Methyl Esters of Liquid Saturated Acids from Phosphatide of Phytomonas tumefaciens

Fraction No.	Jacket temperature	Weight	M. p.	Refractive index	Fraction No.	Jacket temperature	Weight	M. p.	Refractive index
	°C.	gm.	°C.	n_D^{25}		°C.	gm.	°C.	n_D^{25}
1	110	0.102		1.4415	12	145	0.070	27-29	
2	114	0.047	10-20		13	150	0.067	26-29	
3	114	0.083	26		14	153	0.049	25-26	
4	114	0.096	14-18	1.4438	15	153	0.104	7-11	1.4490
5	114	0.065	0		16	165	0.096	4- 9	
6	135	0.034	18-23		17	165	0.039	0	
7	137	0.045	21-24		18	167	0.313	<0	1.4500
8	138	0.080	27-28		19	167	0.245	<0	1.4500
9	139	0.029	29-30		20	167	0.331	<0	1.4500
10	140	0.023	33		21	174	0.125	8	1.4526
11	145	0.060	30-32		22	200	0.123	12	1.4562

The combined constant boiling fractions (Nos. 18, 19, and 20) were subjected to a very slow redistillation and twelve cuts were taken. The first and last fractions, which weighed only a few mg. each, deviated slightly in index of refraction and were discarded. No further inhomogeneities could be detected by redistillation. Since the resolving power of the still was about nine theoretical plates, the presence of an appreciable amount of homologous impurity could readily have been detected. The possibility that the main fraction contained branched isomers differing by only a few degrees in boiling point cannot be excluded by this distillation. The analysis of the methyl ester was as follows:

Analysis— D_4^{25} 0.8878, n_D^{25} 1.4500, $[\alpha]_D^{25}$ 0.00 (8.1 per cent solution in ether). 6.708 mg. methyl ester: 19.05 mg. CO₂ and 7.82 mg. H₂O

C₂₁H₄₂O₂. Calculated, C 77.30, H 12.89; found, C 77.44, H 12.94

The Free Acid—A portion of the ester was saponified by refluxing for 3 hours with an excess of alcoholic potassium hydroxide. Most of the alcohol was evaporated under reduced pressure, and the residue was dissolved in water and extracted with ether to remove any traces of unsaponified material. The aqueous soap solution was then acidified and the fatty acids were extracted with ether. The ethereal extract was washed with water until the washings were neutral to litmus, after which the ether was evaporated. The free acid crystallized on standing for some time at 20°, and melted at 24°. The melted acid recrystallized slowly on cooling.

Analysis—5.549 mg. acid: 15.63 mg. CO₂ and 6.391 mg. H₂O

C₂₀H₄₀O₂. Calculated, C 76.92, H 12.82; found, C 76.80, H 12.80

Titration—37.47 mg. acid required 7.31 cc. 0.01654 N KOH; neutral equivalent 311, calculated for C₂₀H₄₀O₂ 312

The Hydrazide—90 mg. of the methyl ester were dissolved in 1 cc. of ethanol, 0.4 cc. of 85 per cent hydrazine was added, and the solution was refluxed for 3 hours. The reaction mixture was diluted with water and extracted with several small portions of ether, and the extract was washed first with dilute hydrochloric acid and then with dilute potassium carbonate and with water. After the ethereal solution had been dried over sodium sulfate, the ether was evaporated. Crystals were obtained by subliming the white waxy solid in a small molecular still at a pressure of 0.0004 mm. The white micro platelets showed strong extinction when rotated between crossed nicol prisms. The melting point was 56.6° and the yield was 93 per cent. When a solution of the hydrazide in dilute methyl alcohol was allowed to evaporate slowly, large exceedingly thin crystals having a pearly luster were deposited, which also melted at 56.6°.

Analysis—12.56 mg. hydrazide required 3.87 cc. 0.0200 N HCl

C₂₀H₄₂ON₂. Calculated, N 8.59; found, N 8.62

It was necessary in carrying out the Kjeldahl digestion to carry out a preliminary digestion with hydriodic acid (6).

Minor Fractions—Because of the small amount of material in the lower fractions of the fore run a pure acid could not be obtained from them. Fractions 6 to 14 contained increasing, then decreasing, amounts of a solid acid which appeared to be stearic acid. The acid obtained from Fraction 10 by saponification and recrystallization from methyl alcohol and acetone melted at 65° and had a neutral equivalent of 288 (stearic acid m.p. 69°, neutral equivalent 284). This acid was apparently carried along in the ether-soluble lead salt fraction by mixed salt formation between the bivalent lead and the branched acids. Fractions 21 and 22 contained higher boiling material which from the low melting point appeared to contain branched components. They were combined and saponified. The free

acid so obtained was a waxy solid melting at 28–30°. It had a neutral equivalent of 330.

SUMMARY

1. The major branched acid in the phosphatide of *Phytomonas tumefaciens* has been obtained in apparently pure form and shown to have the composition $C_{20}H_{40}O_2$. The name "phytomonic acid" has been proposed.

2. The physical properties of phytomonic acid indicate a branched chain structure.

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DETERMINATION OF SERUM CALCIUM BY PRECIPITATION WITH OXALATE

A COMPARATIVE STUDY OF FACTORS AFFECTING THE RESULTS OF SEVERAL PROCEDURES

By JULIUS SENDROY, JR.

(From the Department of Experimental Medicine, Loyola University School of Medicine,
and Mercy Hospital, Chicago)

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Most of the methods for analysis of serum calcium have been based on the principle of precipitation and isolation of calcium as the insoluble oxalate. In the successive analytical steps involved, (a) the preparation of the serum sample for (b) its precipitation as calcium oxalate, (c) the isolation of the pure precipitate, and (d) its quantitative measurement, many variable factors may affect the results and must be empirically controlled.

The final step (d), the measurement of the calcium, usually and most conveniently as oxalate, can be made the least variable and most accurate part of the analysis. The dissolved oxalate can be estimated by titration with potassium permanganate or ceric sulfate, or by the gasometric method of Van Slyke and Sendroy (43, 45, 36), with an error of 0.5 to 1 per cent. The washing of the calcium oxalate precipitate in the course of its isolation (c), usually by centrifugation, is a strictly empirical and arbitrary procedure (6, 4, 45, 49, 35), which can nevertheless be carried out to yield constant, reproducible results. Error in this step alone can be limited to a maximum of 1 per cent.

Concerning the initial steps (a) and (b) there is much less certainty. The serum calcium may be precipitated by the addition of oxalate (Procedure 1) to serum *directly* (without further treatment other than dilution with water), as proposed by Přibram (32) in 1871, and others (47, 5, 6, 17); (Procedure 2) to the dissolved *ash* of serum, as applied to microanalysis by de Waard (46) and Kramer and Howland (16); or (Procedure 3) to a *protein-free filtrate* of serum, as was first done by Lyman (22) and Halverson and Bergeim (9).

Were the results obtained under such widely different conditions of precipitation equal in accuracy, the method of choice would be determined largely by convenience or the amount of sample available. However, when comparative analyses of the same serum samples are carried out in the three ways mentioned, the method of washing and measuring the oxalate being the same throughout, large differences in results may be found, in the same laboratory and by the same analyst. Despite many studies

devoted to a consideration of such discrepancies, there is no agreement as to the optimum conditions for the quantitative precipitation of serum calcium as oxalate, nor has a satisfactory explanation been found to reconcile the differences in results obtained by various methods, and by different investigators.

Indicative of the situation are the following references to comparative investigations in which the preparation of the sample and the conditions affecting the precipitation of serum calcium were studied as variable factors. Results by ashing and by direct precipitation have been found to be the same (32, 46, 47, 6, 10, 2, 29, 39), higher for direct precipitation (3, 50, 48), and also lower (30, 11, 41, 2). In comparison with results obtained by ashing, the deproteinization methods have been reported to give values the same (18, 26), higher (9, 6, 38, 20, 10, 40, 29), and also lower (6, 19, 2). Higher values for calcium of trichloroacetic acid filtrate compared with directly precipitated serum calcium have been found (45, 40, 29, 12) and likewise, lower ones (6, 2). Přibram (32) and Blühdorn and Genck (1) could find no calcium in the ash of serum from which calcium had been directly precipitated, nor could de Waard (47) find any in the filtered serum after direct precipitation. Van Slyke and Sendroy (45), however, found calcium in the trichloroacetic acid filtrate of decalcified serum.

Since the papers cited embrace a wide variety of techniques for each of the four steps in analysis mentioned above, no inferences even as to the general trend of the values for any group of similar procedures may be drawn. Obviously, conclusive evidence concerning the validity of these conflicting groups of results, and an explanation of the origin of the discrepancies involved, are desirable (25).

The accuracy and precision of the gasometric method for calcium (43, 45) were well suited for the purposes of the present investigation. The maintenance of a uniformity of sample size, of washing technique, and of calcium oxalate measurement eliminated these factors as variables in a study concerned mainly with the factors governing the precipitation of serum calcium in different media and under various conditions. Control analyses of known salt solutions, paralleling those of serum at every step, and for each of the factors studied, served as standards of absolute accuracy. The results indicate that it is possible to obtain complete agreement in analyses of the same serum sample, whether calcium is precipitated directly from the serum, from the ash, or from the deproteinized filtrate, *provided that appropriate corrections are made in each case*. Many of the discrepancies reported in the literature are merely artifacts, arising from an accumulation of errors, no one of which is ordinarily detectable in blank analyses by the usual procedures heretofore followed.

Procedure

The techniques used, with some modifications of our own, were similar to ones described in the literature. For the sake of clarity, and because some of the discrepancies cited may owe their origin to certain details of procedure, an exact description of the analytical methods employed is given in the following.

(a) Preparation of Serum Sample and (b) Precipitation of Calcium

With few exceptions, all analyses were performed in triplicate, the samples used being 1 cc. of serum (or its equivalent). The sample, in a conical bottom 12 cc. centrifuge tube, was distributed in a fluid volume of 6 cc. in every analysis. The calcium was precipitated, at room temperature, by the addition of 1 cc. of saturated ammonium oxalate. Precipitation ("digestion") took place overnight, or for a period of about 16 hours.

*The blanks for all analyses reported in this paper were uniformly prepared by the addition of 1 cc. of saturated ammonium oxalate to 6 cc. of distilled water.*¹

Procedure 1. Calcium Precipitation Directly in Diluted Serum—To 1 cc. of serum, there were added 5 cc. of distilled water, followed, after mixing, by 1 cc. of saturated ammonium oxalate. The contents of the centrifuge tube were thoroughly mixed.

Procedure 2. Calcium Precipitation in Dissolved Ash of Serum—To 1 cc. of serum in a 15 cc. platinum dish, 2 cc. of 1 N HCl were added; the mixture was evaporated to dryness on a steam bath, then placed in an oven at 110° for 15 to 20 minutes. The dish was transferred to a muffle furnace initially at the same temperature. The furnace was gradually heated to 500°, at which temperature the sample was then ashed for 2 hours.² After cooling, the ash was dissolved in 2 cc. of 1 N HCl and the solution evaporated on a steam bath.³ The dish was again cooled and the ash dissolved in 2 cc. of 1 N HCl. The solution was quantitatively transferred to a centri-

¹ These "water" blanks served (a) to correct for possible failure to remove ammonium oxalate completely in washing, and (b) to provide necessary corrections for the gasometric measurement (44, 36).

² If the furnace is kept at 500° for the duration of the day's work, the initial heating of the dried serum must be done with especial care to prevent mechanical spattering of the ash as water of combustion is evolved. With the furnace door open, the dish is held by tongs and alternately moved in and out of the furnace until the fumes, which should be evolved from the material gently, are no longer visible. The dish is then heated at 500°.

³ A white ash is usually obtained at this point. On the rare occasions when visible particles of carbon remain, the ashing at 500° should be repeated for $\frac{1}{2}$ to 1 hour.

fuge tube, followed by three portions of 1 cc. of water used to wash the walls of the platinum dish.

To the 5 cc. of solution in the centrifuge tube, 1 cc. of freshly prepared 20 per cent sodium acetate, 2 drops of 0.1 per cent brom-cresol green indicator, and 1 cc. of saturated ammonium oxalate were added (45). After the mixture was stirred with a thin, footed glass rod, a few drops (usually 6) of 1:1 ammonia were added to obtain a color matching that of a similar volume of 0.067 M KH_2PO_4 solution, of pH about 4.5, containing the same amount of indicator. The stirring rod was washed off into the tube with a few drops of water.

*Procedure 3. Calcium Precipitation in Protein-Free Filtrate of Serum—*To 1 measured volume of serum in a volumetric flask of 5-fold volume, were added 3 volumes of water, then, drop by drop, with gentle whirling, 1 volume of freshly prepared 20 per cent trichloroacetic acid solution (45). After the addition of a drop of caprylic alcohol, and water to the mark, the material was mixed and allowed to stand $\frac{1}{2}$ hour. The mixture was then centrifuged for 10 minutes at 2200 R.P.M. The supernatant fluid was filtered through a sintered glass filter funnel, Jena No. 3G4, No. 3G3, or Pyrex No. 30F. Samples of 5 cc. of filtrate were transferred to centrifuge tubes and the calcium precipitated exactly as described above for 5 cc. volumes of dissolved ash. Usually, less (about 2 drops) 1:1 ammonia was needed to obtain a final pH of 4.5.

(c) *Purification and Isolation of Precipitated Calcium Oxalate*

The procedure followed was a modification of that previously described ((45) p. 221). After standing for at least 16 hours,⁴ samples and blanks alike were centrifuged for 5 minutes at 2600 R.P.M. By means of an up-turned capillary, the tip of which was held below the surface of the liquid, all but 0.2 to 0.3 cc. of the supernatant fluid was slowly siphoned off. The precipitate was washed with 2 per cent ammonia water⁵ as follows: 1 cc. was slowly admitted from a pipette to wash the entire inner surface of the tube, which was then slanted while an additional 2 cc. were allowed to flow in quickly. The supernatant wash fluid was then carefully stirred with a thin, footed glass rod, the end of which was permitted to come no nearer than within 0.5 cc. of the bottom of the tube.⁶ Prior to withdrawal from

⁴ Bull's (3) results probably explain the variation in the time required for direct precipitation by different workers. He found that while calcium could be completely precipitated in some sera in a half hour others required at least 16 hours, depending on the viscosity and protein content of the sample.

⁵ The ammonia wash water should be freshly prepared for each day's use, from the concentrated material (21).

⁶ This washing procedure avoids disturbance of the precipitated calcium oxalate by a violent inrush of wash water, which nevertheless is well mixed with the 0.2 to 0.3

the tube, the rod was washed off with a few drops of ammonia water. After 5 minutes centrifugation at 2600 R.P.M., the washing, centrifugation, and withdrawal of fluid were repeated twice as above, except that the supernatant wash fluid was not again stirred with the glass rod.

(d) *Quantitative Measurement of Precipitated Calcium Oxalate*

The gasometric procedure of Van Slyke and Sendroy ((45) pp. 221-224) was followed, modified as described elsewhere (36). The *c* correction was obtained from the analysis of the *blank*, prepared, washed, and analyzed as above.¹

EXPERIMENTAL

Analyses of Salt Solutions of Known Calcium Content

Salt solutions of known calcium content were analyzed under conditions of controlled accuracy, *by the same techniques as are outlined above for serum samples*. The dilute solutions used, approximating the inorganic pattern of serum, were prepared from stock solutions at frequent intervals, exactly as previously described ((35) p. 253), and contained, in milliequivalents per liter, CaCl_2 5.0, MgCl_2 3.0, NaCl 154, KH_2PO_4 1.2. Occasionally magnesium or phosphate, or both, were omitted. In this work, sixteen stock salt solutions were used over a period of 8 months.

The results are summarized in Table I. All values obtained are included in the calculations. The statistical treatment follows methods described by Mainland (24); the *t* and *x* tables of Fisher (8) are used as criteria of significant differences.

Series 1. Analyses by Direct Precipitation—The mean of 5.01 milliequivalents of calcium per liter indicates the accuracy and reliability of this technique. The difference of the mean by +0.01 milliequivalent from the known value is not statistically significant.⁷

Series 2. Analyses by Precipitation from Dissolved Ash of Sample—(a) These were carried out as described for serum. The difference of the mean by +0.11 milliequivalent from the known value is highly significant (Table I).

(b) In another group of analyses, actual ashing was not performed, but

cc. of supernatant fluid left above the crystals. Mixing tests with colored solutions and periodic analyses of known calcium solutions (Table I) have established the correctness of this empirical procedure.

⁷ As in all analyses of salt solutions reported in Table I, the calcium in these samples, also, was precipitated at pH 4.5. This adjustment, not made when serum calcium was directly precipitated, followed the addition of oxalate, and usually required the addition of 1 drop of 1 N HCl.

the calcium in 1 cc. samples was precipitated in the presence of all of the reagents used in the ashing procedure. A mean of 5.13 milliequivalents of calcium per liter was obtained.

The difference of 0.02 milliequivalent between the means of groups (a) and (b) might be attributed to the elimination in the ashing group (a) of some organic contaminant reacting with $\text{Ce}(\text{SO}_4)_2$ to produce CO_2 . However, the difference here is statistically not significant. It is clear that

TABLE I

Results of Analyses of (Serum-Salt) Solutions Containing 5.00 Milliequivalents of Calcium per Liter

The results are given in milliequivalents per liter.

	Series 1, direct pptn.	Series 2a, ashing	Series 2b, reagents for ashing	Series 3a, CCl_3COOH "filtrate"	Series 3b, CCl_3COOH "filtrate" ashed
No. of analyses	46	9	20	33	11
Mean ($= M$)	5.01	5.11	5.13	5.18	5.16
" error ($= \Delta M$)	+0.01	+0.11	+0.13	+0.18	+0.16
" deviation	± 0.040	± 0.032	± 0.049	± 0.057	± 0.046
Standard deviation	± 0.047	± 0.042	± 0.059	± 0.069	± 0.065
" error of mean ($= \epsilon_M$) ...	± 0.0069	± 0.0140	± 0.0132	± 0.0120	± 0.0196
$t = \Delta M / \epsilon_M$, referred to known ...	1.45	7.9	9.9	15.0	8.2
Standard error of difference ($= \epsilon_D$) between means, referred to Se- ries 1		± 0.0154	± 0.0144	± 0.0138	± 0.0208
$x = \Delta M / \epsilon_D$, referred to Series 1 ...		6.5	8.3	12.3	7.2
Standard error of difference ($= \epsilon_D$) between pairs of means	Significance ratio of t or x $= \frac{\Delta M}{\epsilon_D}$		Pairs of means compared		
± 0.0219	$t = 0.91$		Series 2a and 2b		
± 0.0230	$x = 0.87$		" 3a "	3b	
± 0.0178	" = 2.81		" 2b "	3a	
± 0.0249	$t = 2.01$		" 2a "	3b	
± 0.0219	$x = 2.28$		" 2a + 2b and 3a + 3b		

the average error of +0.12 milliequivalent (for (a) and (b)) obtained by the ashing technique is caused by the use of those additional reagents not required in the direct precipitation method.

Series 3. Analyses by Precipitation in Presence of Reagents Used for Protein-Free Filtrates—(a) These were carried out as described for trichloroacetic acid filtrates of serum. Since no protein was present in these samples, the centrifugation and filtration through sintered glass were

omitted after it had been shown that these steps *per se* had no effect on the results. The difference of +0.18 milliequivalent from the known value is highly significant (Table I).

(b) In another group, 5 cc. samples of trichloroacetic acid "filtrate" were first dried with 2 cc. of 1 N HCl and *ashed* exactly as described for serum and Series 2a of salt solutions above. A mean of 5.16 milliequivalents per liter was obtained. Statistically, as for Series 2a and 2b, the difference of 0.02 milliequivalent between the means for Series 3a and 3b is not significant. However, both means (5.18 and 5.16 milliequivalents), obtained by the "deproteinization" technique, are decidedly significant in difference from the known value (5.00), or the mean for Series 1. Furthermore, comparison of the means of appropriate unashed (Series 2b and 3a) or ashed (Series 2a and 3b) pairs, and of the combined groups (Series 2a + 2b) and (Series 3a + 3b), indicates that the increase in average error of 0.05 milliequivalent per liter, of Series 3 with trichloroacetic acid, over that of Series 2 without it, is fairly significant. Since the reagents used in both series were otherwise the same, this increase is attributable to the effect of trichloroacetic acid.

Individual Factors Responsible for Errors in Calcium Analyses of Salt Solutions and Serum

The foregoing results have been quantitatively confirmed, by direct, independent experiments designed to determine the various individual factors involved in serum calcium analyses as they have been carried out in the past by ourselves and others.

Effect of Low Calcium Concentration in Sample Analyzed—From the results with serum-salt solutions (Table I, Series 2 and 3), it might reasonably be expected that the calcium-raising effect of the reagents could be eliminated by the use either of reagents previously tested as calcium-free, or of suitable "reagent" blanks instead of the "water" blanks used in these analyses. However, no calcium has ever been detected in our individual reagents by qualitative tests, either in this or in previous work (45), nor have "reagent" blanks ever been found quantitatively different from "water" blanks.

An explanation for the failure to detect small amounts of calcium, as an impurity or otherwise, is afforded by a series of experiments, comprising 1 cc. analyses of twenty serum-salt solutions diluted to contain calcium from 0.1 to 2.5 milliequivalents per liter (2 to 50 per cent of normal serum concentration), performed under the conditions outlined above for direct precipitation (Series 1). Measurements of calcium were made gasometrically (45) and photoelectrically (35). The results deviated from stoichio-

metrical yields for samples containing less than 1.80 milliequivalents per liter, below which concentration they were accurately expressed by the straight line equation,

$$\text{m.eq. Ca per liter sample present} = 0.86x + 0.25$$

where x = milliequivalents of Ca per liter of sample found by analysis. Thus, under the conditions of analysis, *up to amounts corresponding to a concentration of 0.25 milliequivalent per liter of the original sample, no calcium at all is detectable, and therefore none would be found in analyses of "reagent" blanks.*⁸ Calcium as an impurity in the reagents to the extent of 10 per cent of normal serum calcium (0.50 milliequivalent per liter) would appear as an error of only 5.8 per cent in the "reagent" blank. Obviously, to measure the error from reagents, analyses should be made of samples at a concentration *above* 1.80 milliequivalents per liter, by the addition of known amounts of calcium, with "water" blanks for control.⁹

Effect of Filtration through Filter Paper—Early in this work, in comparative analyses of serum, values for trichloroacetic acid filtrates passed through "ashless" filter papers were always higher (from 1 to 24 per cent) than the results by ashing or direct precipitation (Table II, Samples 1 to 20). These observations, which confirmed previous work (45), led to an extensive inquiry into the effect of filtration through filter papers, and the elimination of their use in microanalysis of calcium.

50 cc. volumes of mixtures of known serum-salt solutions and trichloroacetic acid (as in Series 3, Table I) were passed once through a 9 cm. filter paper, then analyzed directly as "filtrate" (a). The filter papers tested were Munktell's No. 00 "ashless," and Whatman's No. 2 and No. 5. Several of the "filtrates" were also analyzed after ashing (b). In such cases the results of groups (a) and (b) were in agreement, indicating the absence of any extraneous organic reduction of $\text{Ce}(\text{SO}_4)_2$ in passage through the filter.

Analyses of such "filtrates," in a test of twenty-four individual No. 00

⁸ Under the conditions of analysis, the actual concentrations in the diluted solutions from which calcium is precipitated will, of course, be one-seventh that of the *original sample*.

⁹ The above empirical results are quantitatively not strictly in accord with theoretical considerations based on the assumption of CaC_2O_4 equilibrium between solid and liquid phases. However, the deviations are in no way related to inadequacies of measurement. Although the calcium in 1 cc. of 0.1 milliequivalent of solution would yield only 3 mm. in the gasometric measurement (45), the photoelectric method (35) is wholly adequate for the determination of corresponding amounts, *i.e.* 2 γ , of calcium precipitated as oxalate from smaller volumes of more concentrated solutions. It is highly probable, since no solid CaC_2O_4 was initially present, that supersaturation, with complete or partial failure to precipitate the calcium present, was responsible for the progressively greater deficiency in stoichiometrical yields from the more dilute samples.

papers selected at random, after correction for the reagent error, gave an average of 0.0016 (± 0.0010) milliequivalent of "extra calcium" extracted per paper. This corresponds to an error of +3.2 per cent in the filtrate analysis of the equivalent of 1 cc. of normal serum. However, two of the papers gave errors of as much as 17 per cent. For two No. 2 papers and four No. 5 papers, the average extractions were 0.0105 and 0.0228 milliequivalent of calcium per paper, respectively.

In other experiments, the filter papers themselves were ashed and analyzed. The yields were 0.0012 (± 0.00025), 0.0112, and 0.0256 milliequivalent of calcium per paper for eight No. 00, two No. 2, and two No. 5 papers, respectively. The ash weight given by the manufacturers, if calculated wholly as CaO, would correspond to +0.0011, +0.0419, and +0.0479 milliequivalent of calcium per paper, respectively.

Thus, there can be no doubt that varying, significant amounts of calcium may be extracted from filter paper by acidified serum filtrates. The failure of previous workers to notice this error is understandable. *A priori*, it would not seem necessary to pass a protein-free "blank" solution through an "ashless" filter paper, especially if the reagents had been tested "calcium-free" in the usual qualitative manner. Moreover, results of this and a preceding section show that even if such control were attempted the error (averaging +3.2 per cent) from most "ashless" papers would be undetectable or inaccurately determined in the usual "blank" analysis, in which errors up to 5 per cent would be completely masked. Finally, since the variability of the individual paper correction, however accurately determined, makes its application impossible, it is clear that *filter paper should not be used in micro calcium analysis*.

Effects of Individual Reagents—To verify the reagent errors indicated by the foregoing results on ashing (Series 2) and protein-free filtrate (Series 3), direct analyses were performed of each of the reagents used, with samples large enough to afford reliable values for calcium.

Samples of 2 cc. of the 1:1 ammonia solution used (6 and 2 drops in Procedures 2 and 3, respectively) were concentrated and dried on a steam bath, in platinum dishes. Analyses of the washings, with known calcium added as for Series 1 in Table I, showed no extra calcium. Samples of 2 cc. of the concentrated HCl from which was made the 1 N HCl used (6 cc. in Procedure 2), were likewise analyzed and showed a small calcium content, of doubtful significance, equivalent to +0.01 milliequivalent per liter for 6 cc. of 1 N HCl. Tests have shown that neither brom-cresol green nor caprylic alcohol affects the results.

Since a maximum error of only +0.01 milliequivalent per liter could be attributed to the other reagents of Series 2, it would seem, from Table I, that an error of +0.11 milliequivalent per liter in calcium values would arise from the use of 1 cc. of 20 per cent sodium acetate. To account more

directly for error from this source alone, 2 gm. portions of the fused crystals ($\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$) were dissolved in 2 cc. of concentrated HCl and water, and the solution evaporated to dryness. Analyses were carried out with known calcium added, as for Series 1 in Table I. Immediately upon the addition of oxalate, a copious white precipitate was obtained. Reacting with $\text{Ce}(\text{SO}_4)_2$, this gave varying yields of CO_2 , corresponding to as much as 100 per cent more oxalate than that expected from the calcium added.

Obviously, this could not be all calcium oxalate. That it represented a coprecipitation effect with a reduction in solubility of ammonium or sodium oxalate was indicated when equivalent amounts of NaCl (0.85 gm.) were similarly treated, *no calcium being added*. 48 hours after the addition of oxalate, a heavy white precipitate was formed, which was then washed several times with absolute alcohol. The material was soluble in water and gave an immediate white precipitate upon the addition of CaCl_2 . This precipitate was insoluble in water, easily soluble in 1 N H_2SO_4 , and was identified microscopically as calcium oxalate.

Thus, at high concentrations of a sodium salt, not necessarily acetate, and in the absence of calcium, large amounts of non-calcium oxalate may be precipitated. At lower concentrations, under the conditions prescribed for stabilizing pH in analyses of ash and of protein-free filtrates, a similar phenomenon may possibly occur, the precipitated sodium or ammonium oxalate escaping solution in the subsequent washing procedure. This would be especially so were the contaminating precipitates occluded to the precipitated CaC_2O_4 . In "reagent" blanks, in the absence of calcium, no trace of extra oxalate has been found. Similar types of contamination by the coprecipitation of sodium oxalate with calcium have been noted (7, 31, 33). Excess cations and acetate have also been cited as complicating factors in calcium analysis (2, 15, 14).

Although the extra oxalate was found only when calcium was precipitated, little calcium could be found in the sodium acetate itself. In a repetition of the above experiments with 2 gm. samples of sodium acetate, with added calcium, the supernatant fluid above the heavy precipitate was withdrawn as usual, and the precipitate dissolved in 1 cc. of 1 N HCl, then reprecipitated overnight. Following this, withdrawal of supernatant fluid, solution, and reprecipitation were again repeated, and the diminished bulk of precipitate washed and analyzed as usual. After correction for HCl and reprecipitation,¹⁰ an increase of +0.23 milliequiva-

¹⁰ In an independent series of analyses, when directly precipitated, unwashed calcium from known salt solutions was dissolved after withdrawal of the supernatant mother liquor, and then reprecipitated, the results were low by 0.10 milliequivalent per liter.

lent of calcium per liter was found, corresponding to a content of 0.02 milliequivalent of calcium per liter as an impurity in the 0.2 gm. of acetate used in procedures of Series 2 and 3. Apparently, in these analyses, an error of +0.09 milliequivalent per liter must be ascribed to the coprecipitation of non-calcium oxalate.

From Table I (Series 3a and 3b), an average error of +0.05 milliequivalent per liter in calcium values would arise from the use of 20 per cent trichloroacetic acid (1 cc. per 5 cc. of protein-free filtrate sample). A direct study of this point was made in analyses of the two lots of trichloroacetic acid used throughout this work. Samples of 50 cc. of 20 per cent solutions were boiled down to 5 to 10 cc., dried and heated on steam and sand baths, and finally ashed in an oven at 500°. The analyses were finished as in Series 2a with known calcium added in some of the determinations. In agreement with results in Table I, the excess calcium found corresponded to a content of 0.04 to 0.05 milliequivalent of Ca per liter, as an impurity in the 0.2 gm. of trichloroacetic acid used in Series 3.

Thus, for our ashing technique, a total correction of -0.12 milliequivalent per liter, or 2.4 per cent of normal serum values, is applicable to all results. The correction covers errors arising from calcium in 1 N HCl (+0.01 milliequivalent), calcium in sodium acetate (+0.02 milliequivalent), and the coprecipitation effect of sodium acetate (+0.09 milliequivalent). For analyses by *deproteinization by trichloroacetic acid*, the error of +0.05 milliequivalent for calcium present in the protein precipitant, added to the error from sodium acetate, makes the total correction -0.16 milliequivalent per liter, or 3.2 per cent of normal serum values. The individual corrections account for, and their sum total is in agreement with, the results recorded in Table I.

Effect of pH at Which Calcium Is Precipitated As Oxalate—The necessity of adjusting solutions to a pH optimum for CaC_2O_4 precipitation in the presence of other possibly interfering electrolytes (27, 28, 37) has often been emphasized. Thus, it has generally been the practice, in ashing and deproteinization procedures, to precipitate calcium oxalate at approximately pH 5.0. However, the factor of pH in the *direct* precipitation of calcium from *serum* has been neglected. One would expect such samples to be somewhat more alkaline than the upper limit of pH 5.6 (37). In fact, by glass electrode measurements of diluted sera (fresh and stale) after addition of oxalate, we have found pH values varying between 7.1 and 7.5. In view of this, and because McCrudden (28) and Shohl (37) carried out their studies under analytical conditions quite different from ours, the effect of pH in our analyses was studied.

In a series of analyses of known serum-salt solutions and sera, calcium

was precipitated directly, at various pH levels between 4.2 and 7.5. The pH values were set by adjustment with dilute HCl or ammonia water, and measured with the glass electrode (23). The calcium values varied only within the limits of analytical error, *without relationship to pH*. Thus, under the conditions of analysis described, calcium may be quantitatively precipitated from serum and from fluids of approximately the same electrolyte pattern as serum, without further adjustment, at pH within the limits of 4.0 to 7.5.

Analyses of Serum Samples

Human serum was obtained from clotted or defibrinated blood freshly drawn from ambulant dispensary patients.¹¹ Samples of 1 cc. or its equivalent were analyzed according to the techniques described ("Procedure"). The corrections found (mean errors of Table I) for the analyses of known salt solutions by Procedures 1, 2, and 3, namely -0.01 , -0.12 , and -0.17 milliequivalent per liter, respectively, were applied to these results for serum. If the individual factors responsible for errors in serum analyses were the same as in salt solutions, corrected results for serum would show complete agreement among the three methods. If otherwise, discrepancies between such corrected results and the true values could then be ascribed to the effect of serum constituents other than the inorganic salts. Since organic constituents are eliminated in the ashing technique, the results (corrected) by that method were accepted as the standard, true values for calcium in serum.

Analyses by Direct Precipitation and by Ashing—For serum, analyses by direct precipitation were higher than by ashing (Table II, Series 1 and 2a), indicative not of an increased precipitation of calcium in the former method, but rather of a further reduction of the $\text{Ce}(\text{SO}_4)_2$ oxidant. The average discrepancy of $+0.04$ milliequivalent per liter ($+0.8$ per cent of normal) for twenty-eight analyses is just barely significant, and is attributable to the precipitation of calcium in the presence of reducing substances present in serum.

That the contaminating effect is not simply the result of an oxidation by $\text{Ce}(\text{SO}_4)_2$ of organic matter of serum retained after washing is indicated by the following. *Serum blanks*, consisting of 1 cc. of serum + 5 cc. of H_2O + 1 cc. of 2 per cent NaCl were compared with blanks of 6 cc. of H_2O + 1 cc. of 2 per cent NaCl. Contrary to previous results with two washings of serum mixtures twice as concentrated ((45) pp. 227-228), *under our conditions of washing* no serum effect was found. The reducing effect of serum occurs, therefore, only when the CaC_2O_4 is precipitated in the pres-

¹¹ We are indebted to Miss Hester E. Reynolds and physicians of the Mercy Hospital-Loyola University Clinics for this material.

TABLE II

Results of Analyses of Calcium in Serum; Values Corrected for Mean Errors Found for Salt Solutions (Table I)

Sample No.	Analysis by ashing, Series 2a	Deviation from ashing (Series 2a) of analysis of		
		Serum by direct pptn., Series 1	CCl ₃ COOH filtrate,* Series 3a	Ash of CCl ₃ COOH filtrate,* Series 3b
	<i>m.eq. per l.</i>	<i>m.eq. per l.</i>	<i>m.eq. per l.</i>	<i>m.eq. per l.</i>
1	4.94	+0.01	+0.49	
2	5.05	-0.07		
3	5.14	-0.02	+0.13	
4	4.96	+0.10	+0.28	
5	4.88	+0.09	+0.19	
6	4.92	-0.03		
7	4.69	+0.08	+0.55	+0.70
8	4.73	+0.11	+0.41	
9	4.73		+0.03	
10	5.00	-0.01		+0.11
11	4.85	+0.13	+0.66	+0.72
12†	4.95	+0.08	+0.14	+0.22
13	5.04	+0.07	+0.16	+0.11
14†	5.04	0.00	+0.26	+0.25
15†	4.88	+0.05	+0.18	+0.21
16	4.95	+0.05	+1.12	+1.24
17†	4.88	+0.01	+0.14	+0.15
18†	4.85	+0.06	+0.29	+0.25
19†	4.85		+0.61	
20	5.00	+0.07	+0.54	+0.50
Average		+0.043	+0.36	+0.41
21	5.04	-0.01	+0.12	
22†	4.84		+0.19	+0.14
23†	4.93		+0.16	+0.12
24	5.16	+0.07	+0.01	+0.05
25	4.89	+0.07	+0.17	+0.17
26	4.99	+0.08	+0.14	+0.14
27†	4.79	-0.02	+0.07	+0.13
28†	4.95	-0.04	+0.09	+0.08
29†	4.96	+0.07	+0.19	+0.15
30†	4.85	-0.02	+0.15	+0.16
31‡	4.91	+0.04	+0.04	+0.08
32	4.69	+0.02	+0.15	+0.12
Average		+0.026	+0.12	+0.12

* The filtrates for the first group (Samples 1 to 20) were obtained by filtration through "ashless" filter paper; for the second group (Samples 21 to 32) by filtration of the centrifuged supernatant through a sintered glass filter.

† Fatty, more or less opaque serum.

‡ Contained 1 per cent by volume of hemolyzed erythrocytes.

ence of serum and is probably caused by occlusion or adsorption of protein on the precipitated crystals.

Analyses by Deproteinization and by Ashing—In the first group (Table II, Series 2a and 3a, Samples 1 to 20), filtrates were obtained by separation from the coagulated protein through Munktell's (9 cm.) No. 00 "ashless" filter paper. In the second group (Samples 21 to 32), filtrates were obtained by centrifugation, and filtration through sintered glass, as described under Procedure 3. In a test of the possibility of error in the precipitation of calcium oxalate in the presence of organic substances derived from serum and present in the trichloroacetic acid filtrate of serum (20, 29), the results for both groups were checked by analyses (Table II, Series 3b) of *ashed* portions of the same protein-free filtrates.

The corrected results show that filtrates through paper (first group) gave widely varying results, all higher (by 0.03 to 1.12 milliequivalents per liter) than the true values found by direct ashing of the serum. The increment (from 1 to 24 per cent) was not only widely different among all the samples, but large variations were sometimes found among portions of the same filtrate poured through different papers. Furthermore, the average increment of +0.36 milliequivalent per liter was not significantly different from that of +0.41 found by ashing the same filtrates. These positive errors, therefore, occur during the steps of deproteinization and filtration, and are not of organic origin, but definitely represent calcium.

In the second group of Table II, the error of the filtrate analyses was reduced, both in range of variation, and in average, to +0.12 milliequivalent per liter, statistically significant in difference from the true, directly ashed values. *Ashing* of the same filtrates, not poured through filter paper, gave values in complete agreement, also indicating an average error of about 2.5 per cent incurred in deproteinization (and filtration). Again, this increment actually represents calcium, and not an organic reduction of $\text{Ce}(\text{SO}_4)_2$ in measurement.

The difference in results for filtrate (Series 3a and 3b) for the two groups of Table II represents calcium acquired as an impurity in filtration through filter paper. The error of +0.12 milliequivalent per liter found when filter paper was not used must be regarded as common to both groups of measurements, and represents an increase in concentration of filtrate calcium incurred in the deproteinization process itself. In direction and in magnitude, this error *corresponds to the effect of volume displacement of fluid by protein precipitate*, as found in other methods in which aliquot portions of protein-free filtrates are used for analysis (42, 34, 13).

Analyses of Previously Decalcified Serum with Known Calcium Added—These experiments served (1) to check the reagent errors for serum due to ashing and deproteinization, *with elimination of the complicating factor*

of volume displacement from the latter, and (2) to prove conclusively that calcium can be completely removed from serum by direct precipitation.

In proportions previously used ((45) p. 228) 2 volumes of serum, 3 of water, and 1 of ammonium oxalate were mixed, and the calcium precipitated overnight in two tubes. The dilute sera were then centrifuged and the supernatant fluids poured through sintered glass filters. To the ash and to the trichloroacetic acid filtrate of portions of this decalcified material equivalent to 1 cc. of serum, were added known amounts of calcium. Analyses were performed according to Procedures 1 and 3. As an additional control, errors due to the deproteinization reagents were also determined. The known salt solutions were analyzed in each case, in the presence of the same reagents used in the analysis of the decalcified serum sample filtrate, exactly as described for Procedure 3a.

TABLE III

Analyses of Ash and Trichloroacetic Acid Filtrates of Previously Decalcified Serum with Known Calcium Subsequently Added (5.00 Milliequivalents per Liter)

The results are expressed in milliequivalents per liter.

Calcium added to	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
I. Ash of decalcified serum.	5.14	5.10	5.12	5.13	5.13	5.28	5.16
II. Trichloroacetic acid filtrate of decalcified serum.	5.16	5.11	5.15	5.08	5.17	5.28	5.19
III. Deproteinization reagent blank.	5.19	5.13	5.08	5.13	5.18	5.32*	5.15*

* Ash of deproteinization reagent blank, + Ca = 5.32 for Sample 6; 5.15 for Sample 7.

Table III constitutes a complete verification of the results presented in previous sections. In Line III, with the exception of Sample 6,¹² the error for deproteinization reagents is in statistical agreement with the average error of +0.16 milliequivalent per liter previously found (Table II). The agreement of results in Line II with those in Line III indicates that the error for deproteinization reagents remains the same, even in the presence of serum protein-free filtrate. Likewise, the results of the ashed serum, Line I, are in statistical agreement with the error for the ashing reagents of +0.12 milli-

¹² We have no explanation for these results, which indicate a consistently high error for the three different types of analyses of Sample 6 carried out the same day. Failure of complete preliminary decalcification of the serum is not a factor, since the same error was found for deproteinization reagents used with salt solution alone (Line III). Use of the same reagents for simultaneous serum analyses (Table II, Sample 30) resulted in little more than the expected average error.

equivalent found for salt solutions (Table I, Series 2a). The results of Table III show clearly that the native calcium in the original whole serum was completely precipitated in the preliminary, direct decalcification.

Procedure 3 for trichloroacetic acid filtrates was also carried out on decalcified serum to which *no* calcium was subsequently added, with filtration through Munktell's No. 00 filter paper instead of sintered glass. The "extra calcium recovered" from these filtrates (from 0.22 to 0.80 milliequivalent per liter) was of the order of magnitude previously found (45), and now proved to represent a summation of filter paper and reagent errors, and not residual calcium unprecipitated from serum by the direct (decalcification) procedure.

DISCUSSION

The foregoing results are indicative of the origin of most of such errors in analyses of serum calcium as occur in the precipitation of calcium oxalate under different conditions. Failure of previous workers to reconcile results by such different methods may be explained in most cases by the fact that some of these errors have hitherto been unrecognized, while others, although anticipated, have escaped detection or adequate control by the usual tests or blank analyses for contaminants. Parallel and comparative analyses of known calcium solutions, however, furnish corrections suitable for each type of analysis and lead to complete agreement in results, whether the serum calcium is precipitated directly, from the ash, or from the deproteinized sample.

Some of these errors are common to all analyses of the same type, regardless of the sample used. Filter papers have been found to contain calcium. Consequently, their use leads to contamination of the sample. Because the error varies from one paper to another, filtrates or supernatant fluids in micro calcium analysis should be centrifuged, or passed only through sintered glass. In our experiments, the conditions of which correspond to those of prevailing techniques, calcium has not been quantitatively precipitated, if at all, when present in concentrations less than 0.26 milliequivalent per liter (1.80 milliequivalents per liter of *original* sample). This observation, therefore, not only accounts for the failure to detect in blank analyses calcium present in small amounts as an impurity in reagents, but also *casts doubt on the validity of most serum calcium values in the literature lower than 3 mg. per 100 cc. of original sample*. It is clear that errors of contamination from reagents, whether from calcium as an impurity or oxalate as a coprecipitate, must be evaluated under actual analytical conditions in the presence of calcium in known, normal serum concentration.

Other errors occur only in serum analyses. Proteins in serum give rise to additional errors not reproducible in, or controllable by the analysis of

known salt solutions. The reduction of the oxidant by protein in the direct precipitation method and the effect of volume displacement in the deproteinization procedure cause positive errors of the order of magnitude of 0.8 and 2.4 per cent, respectively. Correction for these errors may be applied only on the basis of many comparisons of results (all corrected for reagents) with those obtained by ashing. For analysis of serum calcium, although the ashing procedure is theoretically the best for standardization purposes, the method of choice involving the minimum of technical detail and correction for error appears to be the direct precipitation procedure. Analyses of deproteinized serum are the least satisfactory in that a greater number of errors may occur.

That a variation in the foregoing errors, or the occurrence of others not found or considered in this work, may arise from differences in the chemical composition of pathological sera remains a possibility, despite the fact that within the range of pathology presented by clinical material from dispensary patients no such factor of error in any of the methods used has been observed in our experiments.¹³

SUMMARY

A comparative study has been made of the factors of analytical error involved in the precipitation of serum calcium as oxalate, under various conditions. Analyses of known salt solutions and of serum, by direct precipitation, ashing, and deproteinization techniques, indicate the following as sources of error: contamination by calcium from filter paper and reagents and by non-calcium oxalate in coprecipitation, the reducing action of serum in direct precipitation, and the fluid volume displacement of proteins in deproteinization. Together with an explanation of their origin, methods of eliminating these errors or of evaluating them accurately have been presented. The application of such corrections quantitatively reconciles the apparent discrepancies among the three methods. In these analyses, calcium oxalate may be precipitated at pH from 4.0 to 7.5. For both accuracy and simplicity, the technique of direct precipitation is, in most cases, the method of choice for the determination of serum calcium as oxalate.

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¹³ It has been reported that fatty or opaque sera may not be satisfactorily analyzed by the direct precipitation method (Tingey (40)). Our data (Table II), although they reveal no such trend, are too few in this respect for any conclusion. The same may be said of the effect of hemolysis (Table II), to which Muller (29) has ascribed large errors in the analysis of trichloroacetic acid filtrates.

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NOTE ON THE GASOMETRIC DETERMINATION OF OXALIC ACID AND CALCIUM

BY JULIUS SENDROY, JR.

(From the Department of Experimental Medicine, Loyola University School of Medicine, and Mercy Hospital, Chicago)

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Several practical modifications and precautions in the use of the Van Slyke-Neill (4) apparatus for the gasometric analysis of serum calcium (Van Slyke and Sendroy (6), pp. 221-224) have been found advantageous in the performance of many analyses (2). The following departures from the original technique add to the convenience and accuracy of measurement of the isolated calcium oxalate.

After the addition of 2 cc. of hot 1 N sulfuric acid to dissolve the washed calcium oxalate, the centrifuge tube is cooled and the outside rim smeared with hot, molten paraffin, which then solidifies in a thin film. For avoiding loss of solution in decantation, this has been found more satisfactory than vaseline, especially for tubes without lipped rims. Transfer of the acid solution to the cup of the Van Slyke-Neill (4) chamber, with repeated decantation and washing, is then carried out with 5 cc. of water, to bring the volume of solution in the chamber to a total of 7 cc.

The liberation and measurement of CO_2 in the gas apparatus are carried out as previously described, except that 5 drops of saturated $\text{Ce}(\text{SO}_4)_2$ in 1 N H_2SO_4 are used instead of 1 cc. of acidified 0.15 N KMnO_4 . The advantages of using a cerium solution, which may be made from $\text{Ce}(\text{SO}_4)_2$, or preferably $\text{Ce}(\text{HSO}_4)_4$ anhydrous (G. Frederick Smith) ((1) p. 244), are several: indefinite stability, eliminating the need of freshly prepared oxidant; a stoichiometrical yield of CO_2 from $\text{H}_2\text{C}_2\text{O}_4$; a lowering of the c correction for the blank; and greater ease in cleaning the extraction chamber after the addition of the alkaline CO_2 absorbent (1.5 cc. of 5 N NaOH).

For the calculations, Table I of Van Slyke and Sendroy (6) is used, although the conditions of analysis described are different from theirs. Analyses of standard solutions have shown that the yield of CO_2 from oxalic acid reacting with $\text{Ce}(\text{SO}_4)_2$ is practically theoretical, as against the 99.4 per cent previously found with KMnO_4 (6). On the other hand, the increase in total fluid volume (S) from 7.0 to 7.2 to 7.3 cc. requires an increase in the correction factor for unextracted CO_2 (Equation 1, Van Slyke and Sendroy (5)) of about 0.5 per cent. Thus, the net effect of the differences in technique is practically nil, and the original factors (6) apply directly without change.

The necessity of cleanliness and adequate washing of the extraction chamber between analyses must be emphasized. The best technique found is as follows: The basic black precipitate obtained on addition of the CO_2 absorbent is ejected. The chamber is washed with water and three times with 1 N H_2SO_4 . In the last washing, the acid is permitted to drop below the 50 cc. mark, so that the connecting tube to the manometer, down to the beginning of the first bend, is also washed. With the mercury leveling bulb in high position, the acid is then successively ejected through both bores of the stop-cock of the chamber, as the stop-cock is opened and closed twice in each position. The left-hand bore is resealed with mercury.

The washing thus carried out tends to avoid contamination by alkaline carbonate ground in with the stop-cock lubricant and freed in the course of a subsequent analysis. It also clears the chamber of alkali and of the insoluble white residue of mercurous sulfate first formed by reduction of excess oxidant by mercury. Ultimately, owing to its adhesion to traces of lubricant adhering to the glass at the 50 cc. mark, there may be, in spite of all washing, an accumulation of this precipitate at that point. We have found that when this accumulation is sufficiently great the reaction of $\text{H}_2\text{C}_2\text{O}_4$ with $\text{Ce}(\text{SO}_4)_2$, as shown by the CO_2 evolved, is not complete after 3 minutes. Subsequent shakings and rereadings at 1 minute intervals will ultimately yield a value approaching, but not quite attaining, a stoichiometrical result. Experimental verification of this retardation effect, probably the result of further reduction of $\text{Ce}(\text{SO}_4)_2$ by Hg_2SO_4 , was obtained by analyses of known oxalate solutions to which Hg_2SO_4 suspended in 1 N H_2SO_4 had purposely been added, prior to the reaction with $\text{Ce}(\text{SO}_4)_2$.

Failure to obtain constancy in readings following the initial extraction for 3 minutes is a certain sign of an unclean extraction chamber. By washing the chamber with cleaning mixture for each day's use, so that adhering particles of Hg_2SO_4 are removed, all trouble of this kind may be avoided.

In time, the *manometer* of the apparatus often becomes difficult to read, because of the residual ethylene glycol (3) drying agent, which adheres in droplets to the glass of the mercury column. As the mercury loses its wetting contact with the glass, its meniscus becomes less distinct. By the addition of 1 part of Triton NE (Röhm and Haas) to 20 parts of the ethylene glycol, the glass remains clear and clean, while smooth flowing mercury, in intimate contact with it, presents an undeformed, sharp meniscus.

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CONVERSION OF *d*-GLUTAMIC ACID TO PYRROLIDONE-CARBOXYLIC ACID BY THE RAT*

By S. RATNER

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, December 4, 1943)

The metabolism of the unnatural forms of many amino acids resembles that of their natural isomers in so far as both may undergo oxidative deamination to the same keto acid which may then be reaminated. Lysine is exceptional, for the natural isomer is apparently not regenerated once nitrogen has been removed (1), whereas *d*-lysine is so slowly degraded that when an even comparatively small amount is administered much of it is excreted unchanged (2).

l-Glutamic acid is known to be very rapidly deaminated and reaminated, both *in vivo* (3) and *in vitro* (4), but *d*-glutamic acid largely escapes deamination (5). In order to follow the metabolic fate of the *d*-amino acid, an isotopic preparation of *dl*-glutamic acid, containing N¹⁵ in the amino group and deuterium attached to the α - and β -carbon atoms, was fed to rats. As in the case of *d*-lysine, the urine excreted by these animals contained more N¹⁵ than could be accounted for as urea and ammonia (last column, Table I). Most of the isotope present in the urinary urea and ammonia can be attributed to the rapid metabolism of the isotopic *l*-glutamic acid of the racemic compound employed for feeding.

Glutamic acid could be isolated from the urine by precipitation with Neuberg's reagent (6), but only after acid hydrolysis. The product proved to be the pure *d* variety. It contained the same concentration of isotopes as the starting material and was therefore identical with the *d* component of the isotopic *dl*-glutamic acid administered. From the data in Table I it can be calculated that 73 per cent of the ingested *d* form had been excreted. Furthermore, the fact that the concentration of deuterium had not changed after the ingestion and excretion indicates that no reaction involving the hydrogen atoms in the α and β positions of the *d*-glutamic acid had occurred.

The compound actually excreted was readily extracted from the acidified urine by means of ethyl acetate. After purification through the barium salt, it was identified as the dextrorotatory form of pyrrolidonecarboxylic acid which corresponds to the unnatural form of glutamic acid.

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Exclusion of Pyrrolidonecarboxylic Acid As an Artifact—In view of the metabolic significance of the excretion of pyrrolidonecarboxylic acid, the possibility that it may have been an artifact formed during the isolation procedure was tested. From the data secured at 100° by Wilson and Cannan (7), it appears that at pH 5 to 7, though at equilibrium only 2 per cent is present as glutamic acid, the rate of reaction at room temperature would be so slow that only negligible amounts of the pyrrolidonecarboxylic acid would be formed in the time available. At pH 2, where the rate is somewhat faster and equilibrium corresponds to about 90 per cent anhydride, no conversion was observed when, as in the isolation procedure, glutamic acid was extracted continuously for 8 hours with ethyl acetate. The isolated pyrrolidonecarboxylic acid could therefore not have been formed from glutamic acid if this had been present in the urine.

Glutamine is known to be spontaneously converted to pyrrolidonecarboxylic acid in solution. According to Melville (8), at pH 7.8 and 37°,

TABLE I

Distribution of N¹⁵ in Urinary Constituents after Feeding Isotopic dl-Glutamic Acid

The values in the last column were calculated from the total nitrogen of the fractions and their isotope concentration. The animals consumed 477 microequivalents of N¹⁵ excess.

	Total N	N ¹⁵ concentration	Amount of N ¹⁵
	<i>m.eq.</i>	<i>atom per cent N¹⁵ excess</i>	<i>microequivalents</i>
Urea.....	100.0	0.104	104.0
NH ₃	2.4	0.020	0.5
Total N.....	108.3	0.257	278.3

22 per cent is formed in 20 hours, and at pH 1.8 the same degree of splitting was observed by him in 5 hours. The formation of pyrrolidonecarboxylic acid from excreted glutamine may, however, be excluded on the following grounds: (1) Glutamine, because of its free amino group, is precipitated by Neuberg's reagent, whereas pyrrolidonecarboxylic acid is not. (2) No glutamic acid could be isolated from the Neuberg precipitate obtained from the unhydrolyzed urine, but it was secured from the filtrate after hydrolysis. (3) When a solution of glutamine at room temperature and pH 2 was extracted with ethyl acetate for 8 hours, the nitrogen content of the extract corresponded to a 4 per cent conversion of glutamine to pyrrolidonecarboxylic acid. As this value presumably represents the resultant of the rates of formation and extraction, it indicates the extent to which recovery of spontaneously formed pyrrolidonecarboxylic acid might be expected if glutamine were present.

Conditions were arranged in a second feeding experiment so as to mini-

mize possible conversion of glutamine, should it have been excreted. This was done by collecting the urine in tubes kept immersed in solid carbon dioxide. The specimens were allowed to warm up to room temperature only during an 8 hour extraction period. The alcohol-insoluble barium salt obtained from the extracted material contained nitrogen equivalent to 1.03 gm. of pyrrolidonecarboxylic acid and an approximately equivalent amount of barium. From this fraction, 0.60 gm. of pure crystalline material was finally obtained. On the basis of the amount of *dl*-glutamic acid fed and the percentage of *d* isomer excreted, the maximum amount expected would be 1.56 gm. Thus at least two-thirds had been removed by extraction with ethyl acetate. *This is far in excess of what might be formed by spontaneous conversion and must therefore represent preformed compound.*

In spite of the observation (9, 10) that glutathione, when heated in aqueous solution, breaks down to yield pyrrolidonecarboxylic acid, it seems unlikely that the *d*-glutamic acid is excreted as a peptide. Peptides, with which conversion should occur much more slowly than with glutamine, are precipitated by Neuberg's reagent.

Physiological Significance of Pyrrolidonecarboxylic Acid Formation—Free glutamic acid was found by Wohlgemuth (11) to be excreted by rabbits after administration of *dl*-glutamic acid, but it cannot be judged from the description of the experiment whether or not hydrolysis may have occurred. The ease with which *l*(-)-pyrrolidonecarboxylic acid is metabolized when fed to rabbits is in accord with the possibility that it may occur physiologically. Abderhalden and Hanslian (12) found none in the urine after administration, but did observe the excretion of *d*(+)-pyrrolidonecarboxylic acid when the racemic variety was fed. This possibility is strengthened by the work of Woodward and Reinhart (13) who have found that both pyrrolidonecarboxylic acid and glutamic acid are formed in the enzymatic hydrolysis of glutathione, in a ratio which depends on the pH of the medium.

The failure of *d*-glutamic acid to be metabolized can, as with *d*-lysine, be ascribed to its insusceptibility towards *d*-amino oxidase (14, 15). On the other hand, its dehydration *in vivo* is rapid and probably enzymatic.

In the physiological conversion of proline to glutamic acid, pyrrolidonecarboxylic acid may be an intermediate, but no direct relationship has been demonstrated (16).

EXPERIMENTAL

Synthesis of Isotopic dl-Glutamic Acid—This was prepared from α -ketoglutaric acid by catalytic hydrogenation, with deuterium gas and isotopic ammonia as already described (17). The compound contained 4.53 atom per cent N^{15} excess and 12.9 atom per cent deuterium. The latter was shown to be located exclusively in the α and β positions (18, 19).

First Feeding Experiment—Two male rats having a total weight of

587 gm. were maintained in metabolism cages on the customary casein diet (20) to which a total of 1.56 gm. of isotopic *dl*-glutamic acid was added (as the monosodium salt) over a period of 3 days. The animals weighed 597 gm. at the end of the experiment. Quantitative urine collections were made and found to contain 1.516 gm. of nitrogen and 0.257 atom per cent N^{15} excess. Urea was isolated from a small aliquot as the dioxanthryl derivative. A total of 1.40 gm. of urea nitrogen was found to be present, containing 0.104 atom per cent N^{15} excess. The ammonia content was found to be 0.034 gm. and contained 0.070 atom per cent N^{15} .

Of the 278 microequivalents of N^{15} found in the total nitrogen excreted (Table I) 104.5 microequivalents were present in urea and ammonia. Since all but 6 milliequivalents of total nitrogen have been accounted for as urea and ammonia, the unexplained 174 microequivalents of N^{15} must be mainly in the form of some other nitrogenous compound of very high N^{15} concentration. Half of the isotopic *dl*-glutamic acid consumed was the unnatural form and contained 239 microequivalents of N^{15} . Thus 73 per cent (174 of the 239 microequivalents ingested) had been excreted.

Isolation of Glutamic Acid from Urine of First Experiment—The urine was concentrated *in vacuo* to about 30 cc. and filtered. The precipitate formed by addition of mercuric acetate and sodium carbonate (6) was decomposed with H_2S and the filtrate, after removal of mercuric sulfide, concentrated to a syrup *in vacuo*. The residue was dissolved in a small volume of water, the solution was made strongly alkaline with barium hydroxide, and the alcohol-insoluble barium salts were precipitated by adding 3 volumes of alcohol. Barium ion was quantitatively removed from the insoluble fraction, which contained 5 mg. of N; the filtrate was concentrated to 1 cc. and saturated with HCl. No crystals appeared after 2 weeks at 5°, nor after hydrolysis for 3 hours followed by prolonged chilling. The alcoholic mother liquor was, after removal of the alcohol, treated in a similar manner, but no glutamic acid could be isolated either before or after hydrolysis.

The filtrate from the Neuberg precipitation was then investigated. This solution, which was expected to contain the desired material, but no glutamic acid as such, was acidified with HCl and mercuric ion removed as usual. The filtrate was concentrated to a small volume *in vacuo* and heated on a steam bath for 4 hours in 5 N HCl. Excess HCl was removed *in vacuo* and the residue taken up in water. This fraction was treated with mercuric acetate and sodium carbonate and the precipitate treated in the manner described above for the isolation of glutamic acid hydrochloride via precipitation of the barium salt in alcohol. The insoluble barium salt contained 20 mg. of N. After three recrystallizations from 20 per cent HCl, 170 mg. of pure glutamic acid hydrochloride were obtained. It contained 4.32 atom per cent N^{15} excess and 12.55 per cent deuterium

(calculated as free glutamic acid). Nitrogen (Kjeldahl) 7.5 per cent (theory 7.6 per cent); $[\alpha]_D^{20} = -24.9^\circ$ (3.02 per cent in 1 N HCl).

Second Feeding Experiment—Two male rats, total weight 590 gm., were treated as in the first experiment except that 4.16 gm. of ordinary *dl*-glutamic acid were consumed over a 4 day period. The collection tubes attached to the metabolism cages were kept immersed in dry ice continuously and replaced daily. Since half of the amount fed, 2.08 gm., was the unnatural isomer, about 75 per cent of which, according to the results of the first experiment, is excreted, an excretion of 1.56 gm. calculated as glutamic acid may be expected from this experiment.

Isolation of Pyrrolidonecarboxylic Acid from Second Feeding Experiment—The daily urine collection was acidified with dilute sulfuric acid to pH 2, filtered, and extracted continuously for 8 hours with ethyl acetate. The aqueous layer remained at room temperature throughout this period. The oily residue obtained by concentrating the combined extracts *in vacuo* did not crystallize.

Separate experiments with known samples of pyrrolidonecarboxylic acid indicated that though the barium salt of the racemic compound was soluble in alcohol, the solubility of the *l*-barium salt was low enough to permit its use for isolation. The ethyl acetate-soluble product was therefore converted to the barium salt by dissolving in 4 cc. of water and slowly adding powdered $\text{Ba}(\text{OH})_2$ with chilling (to avoid hydrolysis) and stirring. When pH 7 had been reached, insoluble material was removed and 5 volumes of ethyl alcohol were added to the clear solution with cooling. The sticky precipitate was centrifuged off, dissolved in water, and barium was quantitatively removed with sulfuric acid. The filtrate contained 112 mg. of N (8.0 milliequivalents), approximately equivalent to the amount (8.5 milliequivalents) of barium removed. Traces of nitrogen-free organic acids were removed by extraction with ether. The solution was brought to dryness and the residue dissolved in ethyl alcohol. On addition of ether, contaminating pigments precipitated out and were filtered off. The clear solution was allowed to stand at 5° for several days. The resulting 605 mg. of crystalline material were recrystallized three times from alcohol-ether; m.p. $156\text{--}159^\circ$ (uncorrected); N (Kjeldahl) found 10.9 per cent; theory 10.9 per cent; $[\alpha]_D^{24} = +11.4^\circ$ (4.5 per cent in water). From 120 mg., by hydrolysis in 20 per cent HCl for 3 hours, 134 mg. of *d*(-)-glutamic acid hydrochloride, m.p. 202° (uncorrected), were obtained. N (Kjeldahl) found 7.6 per cent; theory 7.6 per cent; $[\alpha]_D^{24} = -24.9^\circ$ (3.37 per cent in 1 N HCl).

SUMMARY

1. Isotopic *dl*-glutamic acid containing N^{15} in the amino group and deuterium attached to the α - and β -carbon atoms was fed to full grown male

rats in small amounts by daily additions to the normal stock diet over a period of 3 days.

2. Approximately 75 per cent of the ingested *d* component was excreted in a form in which the amino group was not free. Optical rotation and isotope analyses of the glutamic acid isolated after hydrolysis have shown it to be identical with the *d* component of the isotopic *dl*-glutamic acid administered.

3. The ability of the rat to convert *d*-glutamic acid into *d*-pyrrolidone-carboxylic acid has been shown by direct isolation of this from the urine.

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STUDIES ON KETOSIS

XXII. THE METABOLISM OF CELLOBIOSE *

By CARROLL E. VANIMAN AND HARRY J. DEUEL, JR.

(From the Department of Biochemistry and Nutrition, University of Southern California
School of Medicine, Los Angeles)

(Received for publication, December 6, 1943)

Although cellulose cannot be utilized in the higher animals with the exception of the ruminants, there are no exact data on whether such hydrolysis products as the disaccharide cellobiose may be available. This sugar differs from maltose only in that it possesses a β - rather than an α -glucoside linkage. No enzyme is known in the gastrointestinal tract which possesses the ability of hydrolyzing the β -glucoside linkage; however, it is possible that this splitting may result from a phosphorysis and that such a reaction can be brought about in the gastrointestinal tract during digestion and absorption.

In the present experiments an evaluation of the absorption of cellobiose has been made by the usual Cori technique. After it was found that cellobiose could be absorbed, a comparison of its glycogenic ability and ketolytic activity was made with glucose given in similar amounts.

Procedure

Studies on absorption and on glycogen formation were made 6 hours after administration of the disaccharide in a 12.5 per cent solution. The reducing sugar was determined by the Shaffer-Hartmann procedure on the filtrate following the precipitation of the proteins by ZnSO_4 and NaOH according to Somogyi, after the gut had been homogenized in the Waring blender. Amytal was used as an anesthetic. The method of Good, Kramer, and Somogyi (1) was used to determine liver and muscle glycogen. Muscle glycogen was estimated on the gastrocnemius muscle which was exposed and frozen *in situ*. The ketolytic activity was determined on rats having an exogenous ketonuria produced by the administration of sodium butyrate in an amount equivalent to 75 mg. (calculated as acetone) twice daily (2). The rats received in addition physiological saline (control group), cellobiose, or glucose in an equivalent volume of solution. The determination of the acetone bodies was carried out by the Van Slyke procedure and

* These data have been presented to the Graduate School of the University of Southern California in partial fulfillment of the degree of Master of Arts by Carroll E. Vaniman.

the usual macro-Kjeldahl method was used for the determination of urinary nitrogen.

Cellobiose was determined by the Shaffer-Hartmann procedure. The comparative equations for the estimation of cellobiose (I) and glucose (II) were as follows:

$$\text{Mg. cellobiose} = 0.284T.D.c + 0.030 \quad (\text{I})$$

$$\text{" glucose} = 0.132T.D.g - 0.015 \quad (\text{II})$$

TABLE I

Comparative Absorption of Glucose and Cellobiose in Fasting Female Rats, Content of Reducing Material in Fasted Rats, and Recovery of Administered Cellobiose in Animals Sacrificed Immediately

	No. of rats	Average weight	Cellobiose					Glucose	
			Fed	Recovered*		Absorbed*		Re-cov-ered	Ab-sorbed
				Method 1†	Method 2‡	Method 1†	Method 2‡		
		gm.	mg.	mg.	mg.	mg. per 100 sq. cm.	mg. per 100 sq. cm.	mg.	mg. per 100 sq. cm.
Fasting controls.....	10	198	0					5.0	
Cellobiose; killed immediately.....	10	194	221.5	217.3 98.2 ± 0.8‡	209.6 94.7 ± 1.1‡			4.8	
Cellobiose; killed after 6 hrs.....	10	220	239.5	137.8	132.2	5.34 ±0.36	5.62 ±0.27	3.2	
Glucose; killed after 6 hrs§..	26								78.3

* Including the standard error of the mean calculated as $= \sqrt{\Sigma d^2/n}/\sqrt{n}$, where d is the deviation and n is the number of experiments.

† Method 1 involves a calculation based on direct application of Equation I, while Method 2 involves the calculation based on Equation III.

‡ Percentage recovery.

§ Experiments of Deuel, Hallman, Murray, and Samuels (6).

where $T.D.c$ and $T.D.g$ represent the titration difference in cellobiose and glucose respectively. In order to determine cellobiose in the presence of glucose in the contents of the gut, it was necessary to determine the titration difference before and after a 90 minute hydrolysis with $N HCl$. The values then were calculated by the following equations obtained mathematically by solution of (I) and (II).

$$\text{Mg. cellobiose (before hydrolysis)} = \frac{0.038(T. D._A - T. D._B) - 0.004}{0.166} \quad (\text{III})$$

$$\text{glucose (before hydrolysis)} = \frac{0.039T. D._B - 0.017T. D._A + 0.007}{0.166} \quad (\text{IV})$$

where $T. D._A$ and $T. D._B$ represent the titration difference after hydrolysis and before hydrolysis (due to cellobiose and glucose).

These formulae were tested empirically in the laboratory with various proportions of cellobiose and glucose and found to give satisfactory results

TABLE II

Liver and Muscle Glycogen of Female Rats Previously Fasted 54 Hours or Given Cellobiose or Glucose 6 Hours Previously

	Body weight	Liver glycogen					Muscle glycogen				
		Per cent*	M. D. S. E. M. D. †	Fisher ‡			Per cent*	M. D. S. E. M. D. †	Fisher ‡		
				Calculated	Significant value‡				Calculated	Significant value‡	
	gm.										
Fasting controls..	206	0.03 ± 0.01 (14)					0.24 ± 0.01 (8)				
Cellobiose..	203	0.38 ± 0.03 (14)	11.07	10.65	2.78		0.30 ± 0.02 (7)	2.68	2.59	3.01	
Glucose....	205	0.52 ± 0.04 (8)	11.87 2.80	14.11	2.84		0.30 ± 0.02 (8)	2.68	2.52	2.98 2.14§	

* Including the standard error of the mean. The values in parentheses are the number of experiments included in the averages.

† Ratio of the mean difference to the standard error of the mean difference when compared with the controls.

‡ Based on a P value of 0.01.

§ Based on a P value of 0.05.

|| Comparison of glucose and cellobiose groups.

when the concentrations were adjusted to give titration differences between 2 and 10 cc. Attempts to determine glucose and cellobiose in a mixture by fermentation of the latter by washed bakers' yeast¹ were unsuccessful, as 6 to 9 per cent of the cellobiose was also fermented. In the presence of glucose as much as 70 per cent of the cellobiose was fermented in a 15 minute interval. Myrbäck (3) has reported that several yeasts including *Saccharomyces fragilia* ferment cellobiose slowly.

Female rats from our stock colony were used throughout because of the lower and more constant level of liver glycogen during fasting (4) and be-

¹ Fleischmann's.

cause of their greater susceptibility to the development of an exogenous ketonuria (5). Cellobiose was obtained from the Eastman Kodak Company. It gave the theoretical specific rotation of $+35.2^\circ$. Pfanstiehl's c.p. glucose was used.

TABLE III

Comparative Ketolytic and Protein-Sparing Action of Glucose and Cellobiose on Exogenous Ketonuria of Fasting Female Rats

The results are expressed in mg. per 100 sq. cm., including the standard error of the mean.

	Average weight gm.	Acetonuria					Urine nitrogen					Fisher <i>t</i>	
		2nd day	3rd day	4th day	Average	M.D. * S.E.M.D.	2nd day	3rd day	4th day	Average	M.D. * S.E.M.D.	Calculated	Significant value†
Controls, 0.9% NaCl	208	53.4 ± 10.1 (10)	76.8 ± 12.0 (10)	88.0 ± 10.8 (9)	72.7 ± 7.1 (29)		43.2 ± 2.3	35.5 ± 1.4	30.0 ± 1.2	36.4 ± 1.4			
Cellobiose, 23.75 mg. per 100 sq. cm. twice daily	202	34.6 ± 7.9 (10)	36.2 ± 8.3 (10)	35.0 ± 8.8 (10)	35.3 ± 4.9 (30)	4.28	38.3 ± 1.9	32.5 ± 1.7	26.5 ± 1.1	32.4 ± 1.3	2.09	2.06	2.58 1.96†
Glucose, 25 mg. per 100 sq. cm. twice daily	200	33.8 ± 5.6 (10)	37.6 ± 5.7 (10)	37.6 ± 5.7 (10)	36.3 ± 3.7 (30)	4.48	42.2 ± 1.6	31.9 ± 1.6	29.1 ± 1.0	34.4 ± 1.3	1.05	1.04	2.58

* Ratio of the mean difference to the standard error of the mean difference when compared with the controls.

† Based on a *P* value of 0.01.

‡ Based on a *P* value of 0.05.

Results

The absorption tests with cellobiose are given in Table I, which also records the value of reducing material in the intestinal contents of fasted rats. Tests are also given of the recovery of cellobiose from the gut when the animals were killed immediately after administration of the sugar.

The amounts of liver and muscle glycogen of female rats which had been previously fasted for 48 hours, or which had received a single administration of cellobiose 6 hours previously after a similar fasting period, or which

had been administered six doses of glucose at hourly intervals after fasting are recorded in Table II.

Table III records the results of experiments in which the ketolytic effect of cellobiose or glucose is compared with an exogenous ketonuria produced by administration of sodium butyrate.

DISCUSSION

The present experiments would seem to indicate that the normal rat is able to utilize cellobiose as a source of carbohydrate. Such utilization is apparently limited by the rate at which the cellobiose is hydrolyzed in the gut.

The proof of the utilization of cellobiose is based on five experimental considerations; *i.e.*, the disappearance of the disaccharide from the gut, the simultaneous appearance of increased liver and muscle glycogen, the demonstration of a ketolytic effect on exogenous ketonuria, and a sparing action on protein metabolism. The effectiveness of the cellobiose is equal to that of glucose. Although slightly greater amounts of liver glycogen resulted from feeding an amount of glucose calculated to be equivalent to the cellobiose digested and absorbed, the difference was not significant statistically. Identical values were found for muscle glycogen and the lowering of ketonuria was the same after equivalent doses of cellobiose and glucose. A somewhat greater protein-sparing action obtains with the cellobiose group, although the differences are not significant statistically.

The rate of absorption of cellobiose is about one-half that of mannose which is 10.2 mg. per 100 sq. cm. per hour. Based on a value of 100 for glucose, this would give the comparative value of 6.8 for cellobiose and 12.3 for mannose.

No glucose was found in the gut following the digestion of cellobiose. This is probably to be explained on the basis that the rate of hydrolysis of cellobiose is so slow that no glucose accumulates in the lumen of the gut.

The fact that there is an identical lowering in ketonuria when similar doses of glucose or cellobiose are given would indicate that the disaccharide is quantitatively transformed to glucose and absorbed as such. Presumably, because of the speed of the reaction (as determined from the glycogen deposition), this hydrolysis takes place in the small intestine. Although some strains of intestinal bacteria are capable of fermenting cellobiose, it is considered improbable that this is responsible for the utilization of the sugar. The formation of alcohol and acetic acids in such fermentations which are not ketolytic would preclude such an explanation. Karrer and Tschan (7) have reported a cellobiase in the snail and it is possible that such an enzyme may be present in the gastrointestinal tract of the rat and that because of its low concentration its presence has been overlooked.

SUMMARY

Cellobiose is absorbed in the rat at a rate of 5.3 to 5.6 mg. per 100 sq. cm. per hour. Its utilization is also indicated by the fact that liver and muscle glycogen are deposited following its absorption, the amounts being approximately the same as when amounts of glucose corresponding to that found from the cellobiose are given. Finally it was demonstrated that these sugars possessed equal ability to lower an exogenous ketonuria. It is believed that this indicates that the utilization of the disaccharide involves the hydrolysis to 2 molecules of glucose rather than a fermentation whereby much of the molecule would be converted to end-products which possess no ketolytic activity.

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A METHOD FOR THE DETERMINATION OF THIOUREA

By LEON C. CHESLEY

(From the Margaret Hague Maternity Hospital, Jersey City)

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The development of an accurate micromethod for the quantitative determination of thiourea in plasma or serum seems important for at least two reasons. (a) There is a possibility that thiourea may be used to measure total body water. Urea is known to be evenly distributed in all of body water, and thiourea may perhaps show a similar distribution. That it may do so is suggested by the work of Purple and Lavietes¹ who gave 0.1 gm. of thiourea per kilo of body weight to three individuals, and from the serum concentrations (10 to 15 mg. per 100 ml.) calculated the apparent volume of distribution as about 67 per cent of the body weight. This, of course, is consonant with the known proportion of the body weight represented by water, although the coincidence may be fortuitous. They were unable to continue with the investigation because of the gastrointestinal reactions to the doses of thiourea required. The more sensitive method here described for the measurement of thiourea would permit the use of well tolerated doses one-tenth of those used by Purple and Lavietes. (b) Thiourea has been used in the treatment of hyperthyroidism, and, as Astwood (1) points out, a method is needed for its determination in blood to permit analyzing more closely its effectiveness and mode of action.

A search of the literature revealed several reactions given by thiourea, but the most promising of these seems to be the color reaction described by Grote (2). In the preparation of his reagent, sodium nitroferrocyanide in alkaline solution is reduced to sodium aquoferrocyanide by hydroxylamine hydrochloride. Treatment of this substance with bromine gives sodium aquoferrocyanide which, on standing in an alkaline medium, is converted to an unidentified compound. This last substance reacts with thiourea to give first a blue color, then purple-red, and finally crimson. Since bromine interferes, it must be removed by aeration in Grote's original procedure.

In adapting this reaction to the quantitative determination of thiourea, we had a great deal of trouble in getting reproducible results. Some of the factors found to influence the reagent were the time intervals between the additions of the successive chemicals, the varying time required for them to dissolve, the amount of shaking in making up the reagent, and the size of the container in which it was made (surface exposed to air?). Since the removal of bromine by aeration introduced a very considerable variable,

¹ Lavietes, P. H., personal communication.

several other methods were tried; finally the addition of phenol was found to be satisfactory. In the modification of the reagent the crimson color reaction was lost, and as the method now stands a blue color is slowly developed. After coming to a maximum, this blue color very slowly fades, but never changes otherwise. Using the Evelyn macro colorimeter, one may read accurately final thiourea concentrations of 1 to 10 γ per ml., corresponding to serum concentrations of 0.4 to 4 mg. per 100 ml.

Reagents—

Approximately $\frac{2}{3}$ N tungstic acid. Equal parts of 10 per cent sodium tungstate solution and of $\frac{2}{3}$ N sulfuric acid are mixed just before use.

Color reagent. Into a 50 ml. Erlenmeyer flask pipette 10 ml. of a 5 per cent solution of sodium nitroferrocyanide, followed by 5 ml. of 10 per cent hydroxylamine hydrochloride. Mix by setting the solution whirling with a single flick of the wrist. After exactly 2 minutes, add 10 ml. of 10 per cent sodium bicarbonate and mix as before. Let stand exactly 10 minutes, at the end of which time add 0.11 ml. of bromine. Mix as before, and let stand for 10 minutes. Then add 5 ml. of 2 per cent phenol. After 10 minutes, dilute a portion of this stock solution 1:20 with 0.05 M phosphate buffer solution at pH 6.0. The reagent is now ready for use. The time intervals between additions of successive chemicals can be varied from that given, but to get a reproducible color reagent, the same schedule must always be followed.

The individual chemical solutions are good for at least 5 weeks, standing in plain glass on the open shelf; the nitroferrocyanide solution will have deteriorated obviously (blue discoloration of flask), but can still be used. Both the stock and diluted color reagents are unstable and are not suitable for use after a few hours. Different lots of the individual chemicals give somewhat different color intensities.

Procedure

Precipitation of Serum (or Plasma) Proteins—1 volume of serum (plasma) is placed in a centrifuge tube, and an equal volume of the $\frac{2}{3}$ N tungstic acid is added. The tube is closed and shaken vigorously. After a few minutes it is filtered, or centrifuged. Some protein will float on top of the supernatant fluid of the centrifugate. To get rid of this, the tube is gently agitated, and a small amount of absorbent cotton is used to wipe down the sides of the tube. The cotton is then pushed down just into the fluid, and the tube is recentrifuged.

Preparation of Urine—Urine is simply diluted with distilled water, to get a concentration of roughly 2 to 10 γ of thiourea per ml. The approximate degree of dilution can be estimated from the following considerations. The renal clearance of thiourea is close to that of urea (3), and the desired con-

centration of thiourea in diluted urine is half that in the serum (because the serum is diluted 1:1 in the protein precipitation). The maximal urea clearance, calculated for urine volumes greater than 2 ml. per minute, is

$$C_m = \frac{UV}{P} \quad (1)$$

where V is reckoned in ml. of urine per minute, P is the plasma (or serum) concentration of urea, and U the urea concentration of urine. Since the average normal plasma clearance of urea (C_m) is 70, and U/P is to be 0.5, the dilution is given by rearrangement of Equation 1 as $140/V$. When the urine volume is less than 2 ml. per minute, the urea clearance is calculated as the "standard clearance," *viz.*,

$$C_s = \frac{U\sqrt{V}}{P} \quad (2)$$

Since the average normal "standard clearance" is 54, the dilution can be calculated as $108/\sqrt{V}$. In patients with diminished renal function, the numerator of the dilution factor may be multiplied by the per cent of normal urea clearance shown by the patient.

Color Development—3 ml. of the serum (plasma) filtrate, or of the diluted urine, are pipetted into a colorimeter tube. To this are added 3 ml. of the diluted color reagent. Appropriate serum and dilute urine blanks are treated in the same way.

Choice of Filter—Spectrophotometric analysis of the colored compound was not made, but a filter having maximum transmission at $580\text{ m}\mu$ was selected for the following reasons. (1) This filter satisfied the logarithmic requirement; *i.e.*, a plot of the logarithms of the galvanometer readings against the concentrations of thiourea gave a perfectly straight line over a wide range. (2) As Fig. 1 shows, the light absorption by the colored compound was maximal over the range of 580 to $620\text{ m}\mu$, as judged from readings taken with different filters. The use of a $600\text{ m}\mu$ filter would improve the sensitivity of the method by only 2 to 3 per cent. This filter was not used merely because it was not available when most of the work was done on the method.

Estimation of Color Intensity—The light transmission is measured in an Evelyn photoelectric colorimeter with a filter having maximum transmission at $580\text{ m}\mu$. The center setting is obtained in the usual manner from the appropriate blank. The No. 6 aperture is used.

Since different lots of color reagent vary somewhat in the rapidity with which they develop maximal color, readings are taken at 10 minute intervals and continued until the galvanometer readings are constant and minimal. In the serum determination, the time required for full color develop-

ment is usually 50 to 70 minutes, but occasionally may be as long as 3 hours. In the urine determination, the time is usually 20 to 40 minutes. The first reading may safely be taken at the minimal times just indicated. In general, the darker the reagent the faster it develops the blue color with thiourea.

Calculation—When the logarithms of the galvanometer readings are plotted against the concentration of thiourea, a perfectly straight line is obtained between thiourea concentrations of 0 and 10 γ per ml. in the final solution, corresponding to serum concentrations of 0 to 4 mg. per 100 ml.

Therefore, calculation of the thiourea concentration may be made from the table of L values ($2 - \log G$). For serum, the thiourea concentration

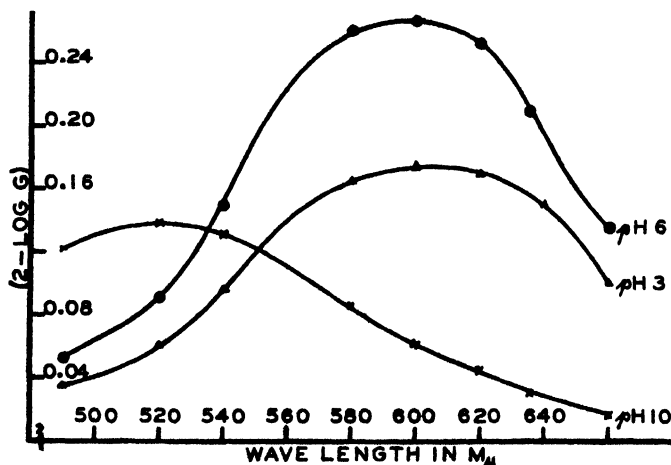


FIG. 1. The light absorption of the colored compounds formed at pH 3, 6, and 10. Each point was obtained by use of a filter allowing maximal transmission at about the wave-length indicated. Each solution had developed maximal color when readings were taken. Thiourea concentration of 1.60 mg. per 100 ml. of pooled serum.

in mg. per 100 ml. of serum (or plasma) is given by $L/0.1640$. In the urine calculation correction must be made, of course, for the dilution factor.

EXPERIMENTAL

Determination of Thiourea Added to Human Plasma, Serum, and Urine—Thiourea was added to pooled plasma (oxalated), pooled serum, and to each of several lots of citrated plasma from single individuals. In each experiment, a fresh lot of stock thiourea was made up, and fresh stock solutions for the color reagent were used. In each experiment, five different plasma or serum concentrations of thiourea were analyzed. In all, ten such experiments were carried out, with seventeen different thiourea concentrations.

The constant, k_2 , was then calculated by dividing each L value by the corresponding thiourea concentration in the serum or plasma sample. The average value for k_2 was 0.1640 ± 0.0003 . The error of single determinations was found usually to be less than 2 per cent. Fig. 2 shows the range and distribution of these errors. In addition to the 50 measurements described above, Fig. 2 also includes seventeen determinations made incidentally in other experiments. For serum or plasma concentrations of less than 1.000 mg. of thiourea per 100 ml. (shown in black in Fig. 2), a slight error in the galvanometer reading makes a fairly large percentage error. For instance, 0.50 mg. per 100 ml. of serum gives a galvanometer reading (G) of 82°. An error of a quarter of a division in G means an error of -1.4

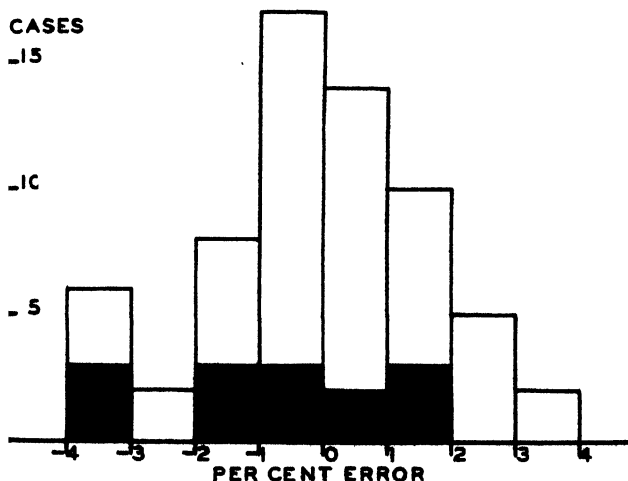


FIG. 2. The frequency distribution of errors in the analysis of sera and plasmas of known thiourea concentrations ranging from 0.2 to 4.0 mg. per 100 ml. The solid black indicates determinations of concentrations of less than 1 mg. per 100 ml.

or $+1.8$ per cent in the final calculation. With a thiourea concentration of 2.00 mg. per 100 ml. of serum, an error of a quarter of a division in G means an error of -0.9 or $+0.6$ per cent.

Similar experiments with urine gave results similar to those with serum and plasma.

Magnitude and Variation of Blank—Sera, oxalated plasmas, and citrated plasmas were obtained from a total of twenty-seven patients. The proteins were precipitated with tungstic acid, and the filtrates treated with the dilute Grote's reagent, as described above in the "Procedure." No color developed in any of these blank sera or plasmas. When compared with blanks made up of water plus dilute Grote's solution or with tungstic acid plus dilute Grote's solution, the serum and plasma filtrates checked within

1 galvanometer division in all but one case. The majority checked exactly with the blank tungstic acid plus dilute Grote's reagent. In one plasma blank, the light transmission was somewhat greater than that of the tungstic acid blank; when the blank read 50° on the galvanometer, this one plasma read 54°.

It may be concluded that the tungstic acid precipitation of proteins removes from blank serum and plasma all substances which give color with Grote's reagent.

In the routine determination of serum thiourea the center setting ranges from 66 to 72, and depends upon the color intensity of the Grote's solution itself, which varies from day to day. (The center setting is obtained by adjusting the light intensity so that the blank serum filtrate plus dilute Grote's reagent gives a galvanometer reading of 100.)

Urines from ten patients were diluted according to the proportions described in the "Procedure." None of these urines developed any color with the dilute Grote's reagent, and all gave readings checking within 1 galvanometer division with blanks made up of distilled water plus dilute Grote's reagent.

Interference by Exogenous Sulfur Compounds—No color was developed by the sulfonamides at the concentrations found in the sera or plasmas of patients under treatment with these drugs.

Sulfocyanate, unfortunately, does develop a strong blue color with Grote's reagent, even at serum concentrations as low as 2 or 3 mg. per 100 ml. This means that sulfocyanate-available water and thiourea-available water cannot be measured simultaneously. Since thiourea does not develop any color in the sulfocyanate determination, the determinations can be made successively, the thiourea-available water being measured first.

Stability of Thiourea in Serum, Plasma, and Tungstic Acid Filtrates—Thiourea seems to be stable for several days, at least, in serum or plasma. Determinations made on serum or plasma which had stood in the laboratory for a week checked with those made at once. In the tungstic acid filtrates the results are often variable after a day or two.

Effect of pH—There are three effects of the acidity of the medium in which color is developed.

(a) When the acidity is greater than pH 3, almost no color appears. From pH 4 to 9, at intervals of 1 pH unit in the experiments, a blue color is developed. Except at pH 9, this blue color does not change, other than to fade slowly after reaching a maximum. At pH 9 the blue gradually changes to pink. At pH 10 a pink color is developed. Fig. 1 shows the light absorption of the colored substances at pH 3, 6, and 10. Acidities from pH 3 to 8 give similar curves, with maximal light absorption between 580 and 620 $m\mu$. At pH 10, the pink color shows maximal absorption at about 520 $m\mu$.

(b) The rate of color development increases with increasing pH, up to pH 9. Fig. 3 shows the color development at pH 4, 6, and 9. Curves for acidities between pH 4 and 8 (except pH 7) are similar to and fall between the curves for pH 4 and pH 6. At pH 7 the curve is very similar to that shown for pH 9.

(c) The maximal color attained varies somewhat with the pH. In aqueous solutions, the maximal color is the same between pH 5 and 8, though the rates of development vary. In the tungstic acid filtrates of plasma and serum, the color development is slightly greater at pH 6 than at any other acidity. Also the color seems to fade somewhat more slowly at pH 6.

Effect of Temperature upon Rate of Color Development—The rate of color development was noted to be much faster in the hot summer months than in the cooler autumn. Detailed experiments upon the temperature effect

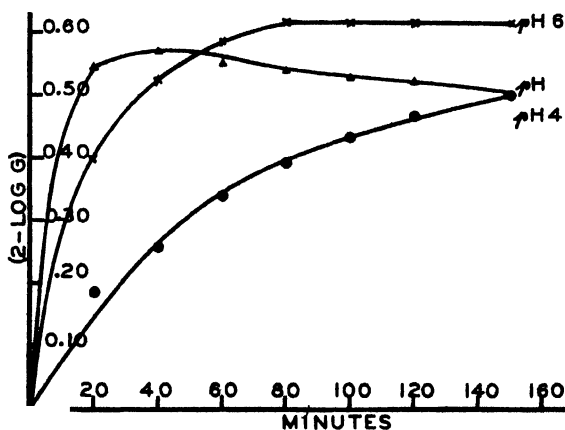


FIG. 3. The rate and degree of color development at pH 4, 6, and 9. Thiourea concentration of 3.75 mg. per 100 ml. of pooled citrated plasma.

were not made. However, parallel determinations made at 21° and at 31° showed that maximal color was attained about twice as fast at 31°.

SUMMARY

A photoelectric method is described for the determination of thiourea at concentrations of 0.4 to 4 mg. per 100 ml. in biological fluids. Higher concentrations may, of course, be measured by dilution of the original samples.

For the determination in serum or plasma, the proteins are precipitated by the addition of an equal volume of approximately $\frac{2}{3}$ N tungstic acid.

In the measurement of thiourea in urine, the urine is diluted according to the rate of urine formation. Working formulae are given for the dilution factors.

A modified Grote's reagent is described, and certain precautions necessary in its preparation are outlined.

A blue color is developed, at pH 6, when 1 volume of modified Grote's reagent (diluted 1:20 in phosphate buffer) is added to 1 volume of serum (plasma) filtrate or diluted urine.

Readings are taken at intervals with the Evelyn photoelectric colorimeter. A filter allowing maximal transmission at 580 m μ is used (Filters 600 or 620 are also satisfactory; all three fulfil the logarithmic requirement). When maximal color has been developed, the reading is recorded for the final calculation.

The error of single determinations in known plasma, serum, and urine concentrations of thiourea was usually less than 2 per cent.

Blank values for serum, plasma, and urine are almost negligible.

The sulfonamide drugs do not give any color, and therefore do not interfere with the measurement of thiourea.

Sulfocyanate does give color with the modified Grote's reagent, and therefore interferes with the determination of thiourea.

Thiourea is stable in plasma or serum for several days.

There is a marked effect of pH in the medium in which color is developed. Best results were obtained by buffering to pH 6 with phosphate buffers.

The rate of color development is accelerated with increase of temperature, at least in the range of temperatures likely to occur in the laboratory.

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AN IMPROVED THIOCHROME METHOD FOR THE DETERMINATION OF THIAMINE IN URINE

By VICTOR A. NAJJAR AND KATHERINE C. KETRON

(From the Department of Pediatrics, the Johns Hopkins University, Baltimore)

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The standard thiochrome procedure for measuring thiamine as developed by Jansen (1) and perfected by Hennessy and Cerecedo (2) involves the adsorption of thiamine on a zeolite column, elution by KCl, treatment with alkali and ferricyanide to convert thiamine into the fluorescent thiochrome, extraction of the latter with isobutanol, and photometric measurement of the fluorescence of the isobutanol extract. A complicating factor in this procedure was discovered by Najjar and Wood (3) in 1940. They reported the presence in urine of a nicotinic acid derivative subsequently designated as F_2 (4), which was adsorbed and eluted together with thiamine and which exhibited greenish blue fluorescence after alkalization and subsequent extraction with isobutanol. F_2 differs from thiochrome in that alkali alone will bring out its fluorescence, whereas alkali and ferricyanide are required to bring out the fluorescence of thiochrome. It would thus appear at first glance that by the use of a blank determination in which alkali alone is added rather than alkali and ferricyanide, and by subtraction of the fluorescence of the blank from that of the sample treated with ferricyanide, the true thiochrome fluorescence could be obtained and the error due to the presence of F_2 eliminated. Another method of eliminating the F_2 error was proposed by Mason and Williams (5). These investigators attempted to eliminate the F_2 error by running a blank determination on a sample treated with sodium sulfite to destroy thiamine. Presumably only thiamine was destroyed by this treatment and the difference between the blank and the thiochrome fluorescence was taken as the true thiamine reading.

It is the purpose of the present communication to show that neither of these two procedures suffices to eliminate the error due to F_2 in the thiochrome determination, an error which in the presence of a small amount of thiamine and of a large amount of F_2 may assume very significant dimensions. We have furthermore now developed a procedure in which this interfering factor is completely eliminated.

The procedure of Mason and Williams is inaccurate because the sulfite treatment employed by them to destroy thiamine also destroys some F_2 , as we have been able to show by measurements on purified F_2 (6) freed from thiochrome. The older procedure of Hennessy and Cerecedo (2), in which the fluorescence of an alkali-treated blank is subtracted from that

of a sample treated with alkali and ferricyanide, we have found to be inaccurate for the reason that the ferricyanide used to oxidize the thiamine to thiochrome also affects the F_2 . The latter, a pyridine derivative, is converted into a pyridone which possesses only 21 per cent of the fluorescence of the original F_2 with the optical system used by Hennessy and Cerecedo. It is obvious that this loss of F_2 fluorescence will have the effect of masking the increase in fluorescence caused by the production of thiochrome. Not infrequently, when the quantity of F_2 is well in excess of that of thiamine, the loss of fluorescence caused by the conversion of the pyridine into pyridone derivative exceeds the gain in fluorescence caused by the conversion of thiamine into thiochrome. Under such circumstances the fluorescence of the alkali blank is actually greater than that of the

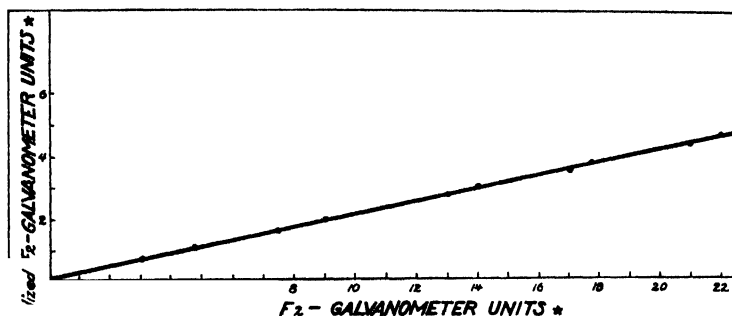


FIG. 1. Relation between fluorescence of F_2 and oxidized F_2 . Each galvanometer unit is equal to $\frac{1}{8}$ Najjar-Wood unit of F_2 , and is derived from $1/26 \gamma$ of thiamine by the optical system of Hennessy and Cerecedo. The linear relation shown does not obtain at concentrations of F_2 greater than 4 Najjar-Wood units, since at these higher concentrations the quantity of ferricyanide employed is insufficient to cause complete oxidation of F_2 .

sample treated with alkali plus ferricyanide, and the thiamine appears to be zero although in fact very appreciable quantities of thiamine may have been present. We have overcome this difficulty by the introduction of a correction factor for the reduction of fluorescence caused by the oxidation of F_2 .

In order to evaluate such a correction factor and to determine whether or not it was constant over a given range of concentration, a study was made of the effect of alkali and ferricyanide upon the fluorescence of substances in the urinary eluate other than thiamine. Studies were made on (1) urine freed from thiamine by treatment with sodium sulfide and subsequently adsorbed and eluted by the Hennessy-Cerecedo procedure, (2) eluates of urine treated with Mallinckrodt's adsorbent charcoal to remove

thiamine and urinary pigments, and (3) on purified F_2 itself. It was found that treatment of each of these preparations with alkali and ferricyanide caused a decrease in fluorescence to 21 ± 0.5 per cent of the original value as measured with the optical system of Hennessy and Cerecedo. The accompanying graph (Fig. 1) illustrates the constancy of this loss of fluorescence caused by oxidation over a considerable range of concentration. The results shown in Fig. 1 indicate that when the fluorescence of F_2 is plotted against that of oxidized F_2 the points form a straight line with a slope of 0.21. In other words, the fluorescence of the oxidized (pyridone) compound is almost exactly 21 per cent of that of the reduced compound in the range of concentration in which measurements are ordinarily made. We have therefore introduced a correction for this effect into the thiamine determination. If the fluorescence of the alkali-treated aliquot is designated A , that of the aliquot treated with alkali plus ferricyanide as AF , and that of the reagent blank as B , the fluorescence of the thiochrome in the aliquot will be represented by the expression $(AF - B) - 0.21(A - B)$.

Thiamine Method

The analytical procedure and optical system employed are essentially those of Hennessy and Cerecedo. A sample of urine containing not more than 3 γ of thiamine (5 to 25 cc. are used depending on the anticipated thiamine content) is adsorbed on a column of freshly activated permutit. The column is eluted with 25 per cent hot KCl until 11 cc. of eluate are collected. Of this eluate two 5 cc. aliquots are taken, one for treatment with alkali alone (3 cc. of 15 per cent NaOH) and one for treatment with alkali plus ferricyanide (0.1 cc. of 1 per cent $K_3Fe(CN)_6$). After such treatment each aliquot sample is immediately extracted with 13 cc. of isobutanol; it is shaken vigorously for 1 to 2 minutes and then centrifuged for 3 minutes at low speed. The isobutanol layer is separated in a separatory funnel; it is then cleared by the addition of a pinch of anhydrous Na_2SO_4 and the fluorescence measured without delay.

Calculation of the thiamine content involves a numerical factor which will vary with the instrument and the procedure used to standardize it, and will include the fraction of the original sample taken as an aliquot for analysis. Our standard of reference is a solution containing 1 γ of thiamine chloride in 5 cc. of KCl, treated with alkali and ferricyanide and extracted with isobutanol exactly as is done with the aliquot of urinary eluate. Thiamine is calculated from the expression

$$\frac{(AF - B) - 0.21(A - B)}{(T - B)} \times \frac{11}{5} = \text{micrograms thiamine in original sample}$$

in which A and AF represent the fluorescence (in galvanometer units) of the aliquots treated with alkali and alkali plus ferricyanide respectively,

B represents that of the reagent blank, and T that of the thiamine standard in these same units. The diaphragm of the instrument is adjusted until the standard solution registers some convenient round number on the galvanometer scale, and the measurements on the unknown are made with this same adjustment.

In practice it is convenient to use a quinine solution for standardizing the instrument each time a determination is made, since quinine solutions are stable. If the relation between the fluorescence of the quinine standard and thiamine standard is known, the instrument can be set at the standard adjustment with the quinine solution. For example, we find that a standard solution containing 20 γ per cent of quinine sulfate gives a fluorescence of 20 galvanometer units, whereas the thiamine standard mentioned above with the same adjustment of the instrument (after deduction of the reagent

TABLE I

Recovery of Variable Amounts of Thiamine Added to Thiamine-Free Urine Eluate Containing Constant Amount of F_2

Thiamine added to sample of eluate	Thiamine recovered	
	Hennessy-Cerecedo procedure	Present procedure
γ	γ	γ
1.0	0.605	1.0
0.75	0.354	0.73
0.5	0.104	0.49
0.25	0.0	0.26
0.175	0.0	0.175

* The eluate contained in each instance 2 Najjar-Wood units per 5 cc.

blank) reads 26 units. Before use, we then adjust the instrument with the quinine standard to read 20 units and employ the following formula.

$$\frac{(AF - B) - 0.21(A - B)}{26} \times \frac{11}{5} = \text{micrograms thiamine in original sample}$$

In order to verify the procedure as outlined above the effect of thiamine additions to a urinary eluate was studied. Urine was freed from thiamine by shaking with Mallinckrodt's adsorbent charcoal which at pH 3 to 3.5 adsorbs thiamine along with urinary pigments. The clear filtrate containing the F_2 fraction was adsorbed on permutit and eluted with KCl. The F_2 concentration of this thiamine-free eluate was 2 Najjar-Wood units per 5 cc. Thiamine in variable quantities was added to this eluate and analyzed by the procedure described above. The results are shown in Table I. It is apparent that the present method permits an accurate

assay of thiamine even in the presence of an excess of F_2 , in contrast to the very incomplete recovery of thiamine if no correction for the oxidation of F_2 is introduced.

TABLE II

Recovery of Thiamine Added to Thiamine-Free Urinary Eluates Containing Varying Amounts of F_2

F_2 present	Thiamine added to thiamine-free eluates	Thiamine recovered	
		Hennessy-Cerecedo procedure	Present procedure
<i>Najjar-Wood units</i>	γ	γ	γ
1.0	1.0	0.80	1.0
2.0	1.0	0.60	1.0
3.0	1.0	0.40	1.04
4.0	1.0	0.21	1.0
1.0	0.5	0.30	0.51
2.0	0.5	0.11	0.50
3.0	0.5	0.0	0.49
1.0	0.25	0.05	0.26
2.0	0.25	0.0	0.24
3.0	0.25	0.0	0.24

TABLE III

Thiamine Concentration of Urine

The values are given in micrograms per 100 cc. By the original thiochrome procedure all values were negative.

Subject	Present modification	Yeast fermentation method (Frey)*
C. G.....	1.3	1.5
C. P.....	1.3	1.4
A. P.....	0.5	0.4
H. K.....	0.7	0.7
G. B.....	2.6	0.7
C. G. (2nd specimen).....	0.8	1.1
C. P. " ".....	2.5	2.0
A. P. " ".....	0.6	0.3

* These measurements were made at the Fleischmann Laboratories through the courtesy of Dr. C. N. Frey.

Table II shows the recovery of an identical amount of thiamine added to a thiamine-free eluate containing variable quantities of F_2 .

As we have pointed out, the circumstances under which this modification is of the greatest value are in the presence of a high nicotinic acid intake or a low thiamine intake, in which case the excess F_2 excretion may mask

thiamine excretion in part or *in toto*. This was impressively demonstrated to us in an experiment we were conducting on human subjects on a thiamine-deficient diet, all of whom were receiving 25 mg. of nicotinamide daily. By the original Hennessy-Cerecedo method each of these subjects gave negative thiamine excretions. However, by the new method it was found that minute amounts of thiamine were being excreted by each subject. Specimens of their urine analyzed by our new procedure were analyzed by Dr. C. N. Frey of the Fleischmann Laboratories using the yeast fermentation procedure. A close agreement between the results obtained by him and by our modified thiochrome procedure was found in all but one of the urines studied (see Table III).

We should like to point out that the correction to the thiamine determination which we have described is applicable not only to urine, but to other biological materials in which F_2 may be present. We have found F_2 to be present in many tissues, notably in liver, and we have also found it to be present in feces.

SUMMARY

A modification of the Hennessy-Cerecedo thiochrome procedure for the determination of thiamine in urine and other biological fluids is described. This permits one to determine thiamine accurately in the presence of F_2 , a derivative of nicotinic acid which may under certain circumstances cause major errors in the thiamine determination.

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3-ACETYL-5,6-ISOPROPYLIDENE ASCORBIC ACID*

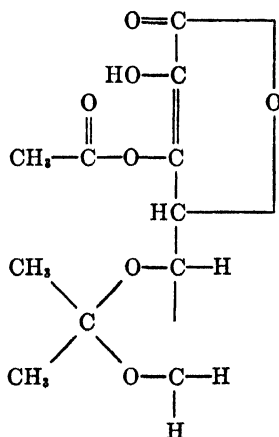
By CARL S. VESTLING AND MILDRED C. REBSTOCK†

(From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana)

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The acetylation of the enediol system of ascorbic acid was undertaken as part of a study of the stabilization of ascorbic acid against oxidation. Previous attempts to prepare an acetyl derivative of ascorbic acid have been recorded (1), but no crystalline compound has been obtained. It appeared that the use of ketene as an acetylating agent would be advantageous in these studies, since the acidic hydrogen attached to carbon atom 3 ought to be readily attacked. It should be noted that 3-acetyl ascorbic acid is a vinylogue (2) of an acid anhydride; hence it would be easily hydrolyzed in aqueous solution.

We have treated the isopropylidene derivative of ascorbic acid with ketene in anhydrous acetone, the progress of the acetylation being followed by indophenol titration. In this way a crystalline compound has been obtained. Its structure has been shown by analysis, quantitative titration with diazomethane, and kinetic studies of the hydrolysis of the material to be of the form shown in the accompanying formula.



This compound is of interest, since it sheds further light on the structure of ascorbic acid. Acetylation of ascorbic acid leads not only to a loss of

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indophenol reducing power but also to a loss of acidic properties. However, in neutral aqueous solution hydrolysis of the acetyl compound proceeds rapidly with the liberation of the 3 position.

Studies with diazomethane (3, 4) have shown that the 3-methyl ether of ascorbic acid is readily prepared by a titration process, whereas the 2,3-dimethyl ether is obtained only by prolonged reaction with an excess of diazomethane (3, 5-7). Therefore, if the 3 position of ascorbic acid (or the isopropylidene derivative) is substituted, reaction with diazomethane should be blocked. With the aid of anhydrous ether solutions of diazomethane which were standardized against benzoic acid, it has been shown that the new compound fails to react readily with diazomethane. This finding offers strong support to the suggested formula.

As a means of substantiating the structure further a study of the kinetics of the hydrolysis of the compound was undertaken. The hydrolysis should proceed according to a pseudo first order mechanism, since changes in the concentration of water would be negligible in dilute solution. This reaction is complicated by simultaneous oxidative destruction of ascorbic acid. As the hydrolysis proceeds, the rate of decomposition of the enediol system gradually overtakes its rate of formation. Hence the reaction must be carried out under conditions which permit an analysis of the hydrolysis in the presence of the competing oxidation. At 70° in 3 per cent HPO_3 at pH 1.9 hydrolysis was found to be complete in 2 hours. The rate of oxidative destruction of isopropylidene ascorbic acid was investigated under similar conditions so that corrections could be applied. However, qualitative evidence indicates that the decomposition proceeds much more rapidly in the hydrolysis system than in the control. In the kinetic study small approximate corrections have been applied to the last two points only. In the later stages of the hydrolysis oxidative destruction becomes the dominant factor, and no experimental or empirical correction was found to be satisfactory.

EXPERIMENTAL

Acetylation of Ascorbic Acid with Ketene at 0° in Aqueous Solution—Preliminary attempts to obtain an acetyl derivative by the use of acetic anhydride with pyridine failed, and the use of sodium acetate resulted in a water-insoluble syrup which resisted all attempts at crystallization.

Accordingly we turned to ketene as an acetylating agent. Ketene was generated in an apparatus similar to that described by Hershberg and Ruhoff (8). It was passed through an ice-salt trap and then through a carbon dioxide-methyl cellosolve trap before being conducted to the reaction system. The material to be acetylated was contained in a 200 ml., 3-necked flask equipped with a mechanical stirrer and a ketene outlet into NaOH.

Ascorbic acid (Mallinckrodt, U.S.P. XI, 0.0607 gm.) was dissolved in 80 ml. of distilled water in a 100 ml. volumetric flask. 10 ml. of glacial acetic acid were added and the system diluted to volume. The resulting pH was 2.3. One-half of the above solution was removed with a pipette and held at 0° as a control. The remainder was treated with a rapid stream of ketene while being kept at 0°. At various time intervals 1 ml. portions were removed and titrated with standardized 2,6-dichlorophenol indophenol. The results of this treatment are shown in Fig. 1. Reaction with ketene effectively blocks the enediol system of ascorbic acid. When ketene treatment is stopped, hydrolysis of the acetyl group proceeds rapidly at 32°, but very slowly at 0°.

Preparation of Acetylisopropylidene Ascorbic Acid—Isopropylidene ascorbic acid was prepared according to Micheel and Hasse's modification (9) of von Vargha's procedure (10). Anhydrous acetone was obtained by dry-

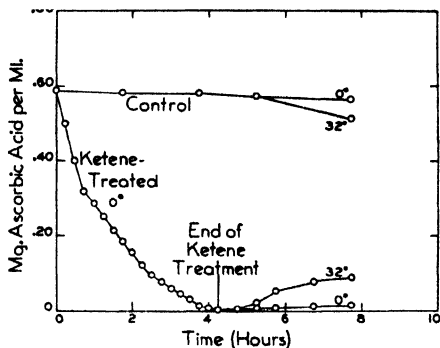


FIG. 1. The acetylation of ascorbic acid in aqueous solution at 0° and pH 2.3. Samples titrated with 2,6-dichlorophenol indophenol.

ing for 12 hours over drierite, filtration, and distillation. Petroleum ether was dried over CaCl_2 and distilled. The use of anhydrous solvents appeared to be important for the success of this preparation.

A sample of isopropylidene ascorbic acid showed the following identifying characteristics: m.p. (corrected) 221.6°, with decomposition (10); $[\alpha]_D^{28.5} = +22^\circ$ (1.8 per cent in distilled water); neutral equivalent 217, calculated 216. Indophenol titration required the calculated amount of the dye within experimental error. A dilute alcoholic solution gave a violet color with ferric chloride. Analysis led to the following values (6, 10).

$\text{C}_9\text{H}_{12}\text{O}_6$ (216). Calculated, C 50.00, H 5.55; found, C 50.24, H 5.76

A solution of 1.23 gm. of 5,6-isopropylidene ascorbic acid in 60 ml. of anhydrous acetone was treated with a rapid stream of ketene. The reaction was carried out at room temperature until the indophenol titration

value was zero (25 minutes). The solution was then allowed to evaporate spontaneously for several hours, and a crystalline material was obtained. This material was dissolved in a little acetone, the solution filtered, and 2.5 volumes of low petroleum ether were added. Long, flat, colorless needles appeared within a few minutes; yield, 56 per cent. The success of this reaction depends on the use of pure acetone derivative as a starting product. A small amount of a yellow syrup is always obtained; it is less soluble than the acetyl derivative and may be easily removed from the acetone-petroleum ether solution. The amount of syrup increases markedly if ketene treatment is allowed to continue after the indophenol end-point has been reached. This suggests that acetylation of the 2 position is probably taking place.

The new compound melted at 115–116° (corrected). A pronounced orange color was obtained with FeCl_3 in ethanol. No reduction of indophenol was observed when the acetyl compound was titrated in anhydrous acetone.

An attempt to obtain a neutral equivalent on the acetyl compound led to the following result. A sample (0.0234 gm.) was dissolved in 25 ml. of CO_2 -free distilled water. Titration with 0.0094 N NaOH required 1.00 ml., phenolphthalein being used as the indicator. A sliding end-point was observed, which undoubtedly indicated hydrolysis of the acetyl group.

Analyses of two different samples of the acetylisopropylidene ascorbic acid which had been recrystallized several times from acetone-petroleum ether yielded the following data.

$\text{C}_{11}\text{H}_{14}\text{O}_7$ (258). Calculated, C 51.16, H 5.43; found (A), C 51.42, H 5.52
" (B), " 51.46, " 5.56

A sample of the acetyl compound (0.2408 gm.) in absolute methanol (15 ml.) showed a specific rotation of $[\alpha]_D^{27} = +27.4^\circ$.

Reactions with Diazomethane—For these experiments diazomethane in absolute ether solution was prepared from methylurea nitrate according to Arndt (11). The ethereal solutions were standardized against benzoic acid as described.

Preliminary qualitative observations showed that 5,6-isopropylidene ascorbic acid in absolute methanol reacted readily with diazomethane, whereas the 3-methyl and 3-acetyl derivatives failed to react. In the latter two cases in which the 3 position is blocked, the yellow color of the diazomethane solution persisted after the first addition of ethereal diazomethane. However, in methanol solution some reaction was always observed as indicated by a slow evolution of nitrogen.

Accordingly, the above observations were placed on a quantitative basis. When the diazomethylations were carried out in absolute methanol at -40°

for 2 minutes before reaction with benzoic acid, it was found that isopropylidene ascorbic acid took up 4.8 moles of diazomethane, whereas the acetyl compound took up 1.27 and 0.58 moles in the case of two samples. Evidently the type of catalytic decomposition of diazomethane in methanol described by Eistert (12) was being encountered. Hence we turned to the use of dioxane as a solvent.

Peroxide-free dioxane (13) was distilled from sodium just before use in these determinations. The procedure was as follows: Isopropylidene ascorbic acid (0.1078 gm.), 3-methylisopropylidene ascorbic acid (0.1000 gm.), and 3-acetylisopropylidene ascorbic acid (0.949 gm.) were dissolved in 5 ml. of dioxane at 13°. The dioxane solutions were then treated with 15 ml. of ice-cold ethereal diazomethane. After 20 to 30 seconds the mixtures were poured into cold 50 ml. portions of ethereal benzoic acid

TABLE I
Quantitative Diazomethylation Studies

Compound	Amount of sample	NaOH for titration	CH ₂ N ₂ reacted with C ₆ H ₅ COOH	CH ₂ N ₂ used per mm compound
	mm	m.eq.	mm	mm
Blank 1	0	3.60	3.68	
" 2	0	3.54	3.74	
			3.71 (Average)	
Isopropylidene ascorbic acid	0.499	4.39	2.89	1.64
3-Methylisopropylidene ascorbic acid	0.435	3.49	3.79	-0.21
3-Acetylisopropylidene ascorbic acid	0.368	3.60	3.68	+0.08

which contained 0.8889 gm. (7.28 mm) of benzoic acid. After 10 minutes at 0° the solutions were titrated with 0.2309 N NaOH to the first definite phenolphthalein end-point. The results, in which no allowance is made for titratable acidity in the resulting methylated ascorbic acid derivatives, are shown in Table I.

The reaction time (20 to 30 seconds) had to be carefully controlled in these experiments, since even in dioxane a slow decomposition of diazomethane was encountered. Nevertheless it seems to be clearly demonstrated that the 3 position is blocked and that the acetyl group is therefore attached to carbon atom 3.

Kinetics of Hydrolysis of Acetylisopropylidene Ascorbic Acid—Preliminary attempts to study the kinetics of the hydrolysis of the acetyl derivative at room temperature and at 0° in 3 per cent HPO₃ or in distilled water did not allow a decisive analysis of the data. A search for more favorable

conditions revealed that the reaction was complete in 2 hours when carried out as described below. The conditions sought were those in which the hydrolysis would be substantially complete before oxidative degradation set in.

Acetylisopropylidene ascorbic acid (18.3 mg.) was dissolved in 3 per cent HPO_3 in a 25 ml. volumetric flask and the system diluted to the mark. The resulting pH was 1.9. When solution was complete, duplicate 1 ml. samples were titrated with standardized indophenol. The flask was then placed in a water bath at $70^\circ \pm 2^\circ$, and samples were withdrawn for analysis at the time intervals noted below. Each sample was immediately cooled in ice on removal from the bath and then titrated within 2 minutes after coming to room temperature. The results are plotted in Fig. 2. As a control system an equimolar quantity of isopropylidene ascorbic acid (15.4

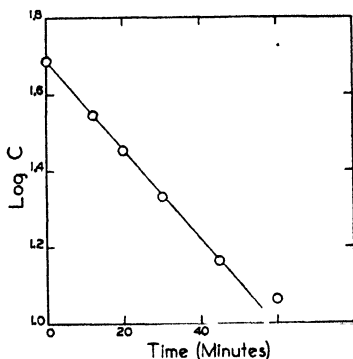


Fig. 2. Hydrolysis of 3-acetyl-5,6-isopropylidene ascorbic acid in 3 per cent HPO_3 at 70° . pH, 1.9. C = concentration of unhydrolyzed material in mg. per 100 ml.

mg.) was treated as above. A linear rate of decomposition of isopropylidene ascorbic acid was encountered during 2 hours. 13 per cent of the material was decomposed in 2 hours, a rate equivalent to 0.1 per cent per minute.

Approximate corrections were applied to the points at 45 and 60 minutes in the hydrolysis experiment. The correction at 45 minutes was 1.7 mg. and that at 60 minutes 2.3 mg. Each correction was based on the assumption that all the hydrolyzed product present at the time of sampling had been undergoing decomposition at a rate of 0.1 per cent per minute since the start of the experiment. Since other observations repeatedly indicated a much more rapid rate of decomposition in the hydrolysis system than in the control, the two points have not been overcorrected.

The plot of the logarithm of the concentration of unhydrolyzed acetyl

derivative against time is characteristic of a pseudo first order reaction, in which the change in concentration of one of the reactants (water) during the course of the reaction is negligible. A plot of the reciprocal of the concentration against time, which yields a straight line in the case of second order kinetics, showed definitely that the reaction did not proceed according to a bimolecular mechanism.

The hydrolysis was about 75 per cent complete in 1 hour. The data obtained during the 2nd hour indicated that oxidative decomposition had set in at such a rate as to make it impossible to correct the values obtained on the basis of the behavior of the control system.

The pseudo first order kinetics of the hydrolysis lend further support to the proposed structure of the acetylisopropylidene ascorbic acid.

SUMMARY

1. A crystalline acetyl derivative of ascorbic acid has been prepared by the action of ketene on isopropylidene ascorbic acid in anhydrous acetone. Its structure has been shown to be 3-acetyl-5,6-isopropylidene ascorbic acid.

2. The ketene acetylation of ascorbic acid in aqueous solution has been studied.

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INTERFERING SUBSTANCES IN THE DETERMINATION OF PHOSPHOGLYCEROL

By G. A. LePAGE

(From the McArdle Institute for Cancer Research, University of Wisconsin, Madison)

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Leva and Rapoport have (1) recently reported a new method for the determination of phosphoglycerol in tissue, and claim that phosphoglycerol constitutes a large part of the ester phosphorus in liver, and to a lesser extent that of other tissues (2). Levels of 27.8 to 28.9 mg. per cent of phosphoglycerol phosphorus (154 to 160 mg. per cent of ester) were reported for livers of fed rats, rabbits, and pigeons (2). This is approximately 25 per cent of the acid-extractable phosphorus of liver. On purely theoretical grounds, there seems to be no reason why phosphoglycerol should be present in such large amounts in resting tissues, as Meyerhof and Kiessling (3) have pointed out. We have been able to account for approximately 78 per cent of the phosphorus of the barium-soluble alcohol-insoluble fraction from rat livers (which would contain the phosphoglycerol) in terms of other esters of known properties. Since this fraction contains 50 per cent of the acid-extractable phosphorus, the values of Leva and Rapoport seemed too high, and it became advisable to examine their new analytical method.

EXPERIMENTAL

Leva and Rapoport oxidize the phosphoglycerol to phosphoglycolaldehyde with periodate, and hydrolyze the product in acid. The inorganic phosphorus released is reported as α -phosphoglycerol phosphorus. Such a degradation has been used on sugar phosphate esters by carbohydrate chemists in order to determine the position of the phosphate group. It is thus known to cause a breakdown of sugar phosphate esters just as occurs in α -phosphoglycerol. Preliminary experiments indicated that ribose-5-phosphate, fructose-6-phosphate, and glucose-6-phosphate all interfere in such a determination of phosphoglycerol. Examination of the fraction used by Leva and Rapoport for phosphoglycerol showed that it contained all three of these interfering esters, which were thus contributing to the phosphoglycerol measurements.

Our tissue samples were all frozen in liquid air (owing to readily available supplies of the material) rather than the dry ice-ether mixture used by Leva and Rapoport. Analyses were made on normal, adult (300 gm.), male, fed rats (Sprague-Dawley, Inc., strain). The rats were anesthetized with nembutal (5 mg. per 100 gm. intraperitoneally) and the livers removed

and quickly dropped in liquid air. The tissue was powdered between chilled steel blocks and in each case transferred to a weighed flask of trichloroacetic acid. The flask was reweighed, the tissue homogenized in a Waring blender, and the débris centrifuged off. Extracts were such that 10 cc. were equivalent to 1.00 gm. of wet weight of liver. The analyses given are averages of determinations on three animals. The extracts were analyzed for inorganic phosphorus and lactic acid, which were found to be 15.9 ± 1.4 and 23.2 ± 2.3 mg. per cent respectively. The tissue was thus demonstrated to be resting liver (Leva and Rapoport found inorganic phosphorus of rat livers used to be 16.7 to 22.8 mg. per cent). Total acid-extractable phosphorus was found to be 111 ± 4 mg. per cent.

Esters Present in Leva-Rapoport Phosphoglycerol Fractions—Aliquots of the liver extracts were carried through the procedure *exactly* as described by Leva and Rapoport (1) for determination of mixed α - and β -phosphoglycerol. The factor 1/0.862 was used to calculate phosphoglycerol phosphorus. A second aliquot was, in each case, neutralized to pH 8.2, and excess barium acetate and 4 volumes of EtOH added. The barium-soluble alcohol-insoluble fraction so obtained was freed of nucleotides and analyzed for phosphoglycerol by the method of Leva and Rapoport. Both of the fractions mentioned above were analyzed for pentose (4) and fructose (5) and, as described later, for reducing sugar (6). Fructose measured $\times 2.39$ gives fructose-6-phosphate as the free acid. Pentose phosphates give the full theoretical pentose equivalent. These points, as well as the equivalents as reducing sugar, have been established with the pure esters. The results, along with the calculated levels of esters present, are listed in Table I. It will be noted that the two fractions show identical properties.

It was necessary to carry out further fractionation in order to measure glucose-6-phosphate and to demonstrate that the sugars measured in the Leva-Rapoport fraction were present as esters rather than as free sugars. Aliquots of the Leva-Rapoport fractions were adjusted to pH 2.0 with HCl and an excess of $\text{Ba}(\text{OAc})_2$ added to remove the sulfate. The BaSO_4 was centrifuged out. The supernatant was in each case treated with more $\text{Ba}(\text{OAc})_2$ plus a small amount of inorganic phosphate and adjusted to pH 8.2. The solutions were left at 0° for 1 hour, and then centrifuged. This removes the inorganic phosphate and quantitatively carries down any hexose diphosphate present. The supernatants from this step were treated with 4 volumes of EtOH, held at 0° for 1 hour, and then centrifuged (the phosphate esters precipitate as barium salts and leave free sugars in solution). The precipitate in each case was treated with a slight excess of H_2SO_4 , the BaSO_4 centrifuged off, and the solution neutralized. Analyses were carried out for pentose, fructose, total reducing sugar, inorganic phosphorus, and total phosphorus. The recovery of organic phosphorus was

92 $\pm$ 1.2 per cent. Recoveries of pentose and fructose were 94 $\pm$ 1.2 and 93.4 $\pm$ 1.5 per cent, respectively. These sugars are thus demonstrated to be present as pentose phosphate and fructose-6-phosphate (not hexose diphosphate). Almost all of the glycogen was lost in the original mercury treatment. Any left was hydrolyzed by a 20 minute hydrolysis in 1 N H₂SO₄ as in the phosphoglycerol treatment and would not precipitate with barium and alcohol. Reducing sugar (corrected for fructose-6-phosphate and pentose phosphate, which give 31.65 and 19.75 per cent of the reduction of an equal weight of glucose respectively) can be calculated as glucose-6-phosphate (reduction 13.2 per cent of that of an equal weight of glucose). The results of these analyses, corrected back to

TABLE I

Analyses on Leva-Rapoport Fraction and Ba-Soluble Alcohol-Insoluble Fraction

Constituent analyzed for	Leva-Rapoport fraction	Ba-soluble alcohol-insoluble fraction,* purified by Leva-Rapoport method
	mg. per cent	mg. per cent
Total P.	32.0 $\pm$ 1.3	32.0 $\pm$ 1.3
Inorganic P.	0	0
Pentose.	33.7 $\pm$ 1.4	33.5 $\pm$ 1.4
Fructose.	10.4 $\pm$ 0.7	10.4 $\pm$ 0.7
Total reducing sugar.	29.65 $\pm$ 1.0	30.2 $\pm$ 1.1
Pentose-5-phosphate P.	6.96 $\pm$ 0.29	6.92 $\pm$ 0.29
Fructose-6-phosphate P.	2.96 $\pm$ 0.20	2.96 $\pm$ 0.20
Glucose-6-phosphate P.	15.0 $\pm$ 1.9	15.5 $\pm$ 2.0
Phosphoglycerol P†.	17.5 $\pm$ 1.2	17.4 $\pm$ 1.2

* Obtained as described by LePage and Umbreit (7).

† With the correction factor 1/0.862 as used by Leva and Rapoport.

100 per cent for the slight losses incurred in fractionating, are given in Table I for both original fractions.

Esters Interfering in Measurements of Leva-Rapoport Phosphoglycerol—Aliquots of the Leva-Rapoport fractions were combined in proportion in order to be able to use the average analyses given earlier. The resulting solution was used in the following experiments. Pure ribose-5-phosphate, fructose-6-phosphate, and glucose-6-phosphate were prepared as previously described (7, 8). Tests were set up as Leva and Rapoport describe, containing portions of the fraction mentioned above. Others were set up with the pure esters added, and with the pure esters only. All tests were in duplicate, and one of the duplicates in each case was not treated with periodate. These were hydrolyzed along with their oxidized duplicates to provide a correction for phosphorus hydrolyzed without periodate oxida-

tion. The results are given in Table II. Each figure is an average of three closely agreeing determinations.

The data in Table II indicate the extent to which these esters interfere with the determination of phosphoglycerol, and demonstrate that the amount of interference is the same whether the esters are in pure form or in the mixture represented by the Leva-Rapoport fraction. Use of these data to correct for interfering esters reduces the value of 17.5 mg. per cent of phosphoglycerol phosphorus to 7.31 mg. per cent, a reduction of 58.3 per

TABLE II
Hydrolysis of Esters with and without Periodate Oxidation

Material used in test	P hydrolyzed after periodate oxidation	P hydrolyzed with no previous periodate oxidation	Amount of added pure ester hydrolyzed and determined as phosphoglycerol
	γ	γ	per cent
Leva-Rapoport fraction*.....	16.9	1.8	
Phosphoglycerol† equivalent to 31 γ organic P.....	26.5	0	85.6
Leva-Rapoport fraction + 20 γ fructose-6-phosphate P.....	33.2	4.7	67.0
Fructose-6-phosphate equivalent to 31 γ organic P.....	25.0	4.4	66.5
Leva-Rapoport fraction + 20 γ ribose-5-phosphate, P.....	31.9	3.4	68.1
Ribose-5-phosphate equivalent to 31 γ organic P.....	23.1	2.4	66.8
Leva-Rapoport fraction + 20 γ glucose-6-phosphate P.....	20.8 *	2.8	14.5
Glucose-6-phosphate equivalent to 31 γ organic P.....	5.9	1.5	14.2

* Equivalent to 31 γ of organic P.

† Equilibrium mixture of α - and β -phosphoglycerol prepared from Eastman mixed esters as described by Leva and Rapoport (1) (heated in acid, precipitated as the barium salt).

cent. The corrected value (7.31 mg. per cent), with the other esters measured, now accounts for 32.23 mg. per cent of phosphorus, or 100.5 per cent of that in the fraction. The animals examined were thus found to have 6.6 per cent of the acid-extractable phosphorus of the liver present as phosphoglycerol, instead of approximately 25 per cent reported by Leva and Rapoport (1).

DISCUSSION

The level of phosphoglycerol phosphorus in rat liver found in this investigation (uncorrected for interfering esters, *i.e.* according to the Leva-

Rapoport method) is much lower than that found by Rapoport, Leva, and Guest (2). However, this point should not be stressed, since Rapoport *et al.* studied a considerably larger group of animals, which may have been under different environmental conditions. The important feature of the experiments here reported is the demonstration that the analytical method of Leva and Rapoport is not specific, and requires correction for three interfering esters, all of which occur in the phosphoglycerol fraction. The studies of phosphoglycerol levels correlated with dietary variations, made by Rapoport *et al.* (2), thus lose their significance for the present, since the results may be due to ribose-5-phosphate or hexose monophosphates. It is not intended that the data presented here be used to make quantitative correction of periodate phosphoglycerol measurements, since the correction so overshadows the analytical figure. A more specific separation or means of determination of the compound should be sought.

SUMMARY

The periodate method of Leva and Rapoport (1) for the estimation of phosphoglycerol is not specific for this material and is interfered with by glucose-6-phosphate, fructose-6-phosphate, and ribose-5-phosphate. The fraction used by Leva and Rapoport for estimation of phosphoglycerol contains all three of these materials in significant amounts.

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MERCAPTURIC ACIDS

IV. THE SYNTHESIS OF *p*-FLUOROPHENYL-*L*-CYSTEINE AND ITS CONVERSION TO *p*-FLUOROPHENYLMERCAPTURIC ACID IN VITRO AND IN VIVO

By S. H. ZBARSKY AND LESLIE YOUNG

(From the Department of Biochemistry, University of Toronto, Toronto, Canada)

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The work described in this paper was undertaken in order to prepare the way for an investigation of the possibility that fluorobenzene is converted to a mercapturic acid *in vivo*. If the metabolism of fluorobenzene is analogous to that of other monohalogenated benzenes (1-3), it is to be anticipated that the urine of animals dosed with fluorobenzene will contain *p*-fluorophenylmercapturic acid. This consideration, together with the fact that no description of *p*-fluorophenylmercapturic acid could be found, led the writers to undertake the synthesis of this compound. This was accomplished by the use of a method based on that employed by du Vigneaud, Wood, and Binkley (4) for the preparation of *p*-bromophenylmercapturic acid and also used by the writers (5) to prepare phenylmercapturic acid. The first stage of the synthesis consisted of the preparation of *p*-fluorophenyl-*L*-cysteine. This compound was obtained by decomposing the product of the interaction of diazotized *p*-fluoroaniline and cysteine cuprous mercaptide. The *p*-fluorophenyl-*L*-cysteine was purified and was then converted to *p*-fluorophenylmercapturic acid by acetylation with acetic anhydride.

The fact that *p*-bromophenyl-*L*-cysteine (4, 6) and phenyl-*L*-cysteine (7) are converted in the rat to the corresponding mercapturic acids made it appear probable that *p*-fluorophenylmercapturic acid is formed in the rat following the ingestion of *p*-fluorophenyl-*L*-cysteine. An investigation revealed that this change does occur. This was established by the isolation of the mercapturic acid from the urine of rats receiving a diet containing *p*-fluorophenyl-*L*-cysteine.

EXPERIMENTAL

Synthesis of p-Fluorophenyl-L-cysteine—This compound was prepared from *L*-cystine (Pfanstiehl Chemical Company) and *p*-fluoroaniline (Eastman Kodak Company) by the following method. To a solution of 5 gm. of *L*-cystine in 100 ml. of 1.5 *N* sulfuric acid were added 3 gm. of zinc dust. The mixture was heated on a water bath for 4 hours and small pieces of mossy zinc were added at intervals. The mixture was filtered while hot

and to the warm filtrate a suspension of cuprous oxide in water was added until no more of the oxide went into solution and the liquid became blue. The solution was cooled in an ice bath and to it was added slowly with stirring a diazotized solution of 4.5 gm. of freshly distilled *p*-fluoroaniline in dilute sulfuric acid. After the mixture had been stirred in the ice bath for 45 minutes, it was heated at 60–70° for 30 minutes and was then filtered. The filtrate was extracted with two 50 ml. portions of ether and was then saturated with hydrogen sulfide. The precipitate which formed was removed by filtration and washed with small portions of 1.5 N sulfuric acid. After the combined filtrate and washings had been heated under reduced pressure to get rid of excess hydrogen sulfide, the solution was cooled and made neutral to Congo red by the addition of ammonium hydroxide. A precipitate which weighed 7.2 gm. was obtained. This material appeared to contain cystine and attempts to obtain pure *p*-fluorophenyl-*l*-cysteine from it by crystallization were unsuccessful. A similar difficulty had been encountered in the preparation of phenyl-*l*-cysteine (5) and had been overcome by subjecting the impure material to reduction. The impure *p*-fluorophenyl-*l*-cysteine was therefore dissolved in 150 ml. of 2 N hydrochloric acid and granular tin was added to the solution. The mixture was heated on the water bath for 2 hours, the supernatant liquid was then decanted, and the residue of tin was washed with several small portions of water. The solution and washings were combined and saturated with hydrogen sulfide, filtered, and the excess hydrogen sulfide was removed from the filtrate under reduced pressure. The solution was neutralized to Congo red by the addition of ammonium hydroxide and a white crystalline precipitate formed. After it had been allowed to stand overnight in the refrigerator, the precipitate was collected, washed with water, and dried *in vacuo* over phosphorus pentoxide. 2.5 gm. of product were obtained which gave the following results when analyzed.

$C_8H_{10}O_2FNS$.	Calculated.	C 50.20,	H 4.68,	N 6.51,	S 14.91
	Found.	" 49.98,	" 4.86,	" 6.37,	" 15.16

The *p*-fluorophenyl-*l*-cysteine decomposed at 180–183° and had a specific rotation of $[\alpha]_D^{22} = +13^\circ$ for a 1 per cent solution in 0.1 N sodium hydroxide.

Conversion of p-Fluorophenyl-l-cysteine to p-Fluorophenylmercapturic Acid in Vitro—0.980 gm. of *p*-fluorophenyl-*l*-cysteine was dissolved in 8 ml. of 1 N sodium hydroxide and the solution was cooled in an ice bath. 1 ml. of acetic anhydride was then added in five portions of 0.2 ml. each. Each addition of acetic anhydride was preceded by the addition of 1 ml. of 1 N sodium hydroxide solution. The solution was stirred throughout the process. It was then allowed to stand for 10 minutes, acidified to

Congo red by the addition of dilute sulfuric acid, and left in the refrigerator for 24 hours. The crystalline precipitate which separated was filtered, washed with water, and dried. This material was taken up in hot ethanol and centrifuged in order to remove a small amount of insoluble matter. The supernatant liquid was poured into warm water and on cooling a crystalline precipitate formed which was collected, washed with cold water, and dried *in vacuo* over phosphorus pentoxide. 0.986 gm. of product was obtained and when analyzed it yielded the following results.

$C_{11}H_{12}O_2N_2S$.	Calculated.	C 51.33, H 4.70, N 5.45, S 12.47
	Found.	" 51.69, " 4.74, " 5.59, " 12.66

The *p*-fluorophenylmercapturic acid melted¹ at 158–159°, and for a 1 per cent solution in ethanol the specific rotation was $[\alpha]_D^{24} = -20^\circ$.

Conversion of p-Fluorophenyl-L-cysteine to p-Fluorophenylmercapturic Acid in Vivo—Two male white rats each weighing about 300 gm. were fed the stock colony diet to which had been added 2 per cent by weight of *p*-fluorophenyl-L-cysteine prepared as described above. The experimental conditions were similar to those described in an earlier paper of the present series (7). The feeding of the diet containing *p*-fluorophenyl-L-cysteine was continued until the rats had ingested 0.900 gm. of the compound. The urine was collected during this period and for 48 hours after the feeding of the compound had been stopped. The urine (80 ml.) was made acid to Congo red by the addition of concentrated hydrochloric acid and was allowed to stand in the refrigerator overnight. It was then extracted with three portions of chloroform each of which was equal in volume to that of the acidified urine. The emulsions which formed during the extraction process were broken by centrifuging. The combined chloroform extracts were dried over anhydrous sodium sulfate, filtered, and the solvent was removed by distillation. The crystalline residue which was obtained was dissolved in a small volume of ethanol, and on addition of water to this solution a light brown crystalline precipitate formed. This material was dissolved in ethanol, and the solution was decolorized with charcoal and filtered. The filtrate was evaporated to dryness and the residue was crystallized from aqueous ethanol. 0.148 gm. of material was obtained which melted at 158–159°. When the compound was mixed with synthetic *p*-fluorophenylmercapturic acid, m.p. 158–159°, the melting point was not depressed. The isolated compound had a specific rotation of $[\alpha]_D^{26} = -20^\circ$ for a 1 per cent solution in ethanol, and on analysis it yielded the following results.

$C_{11}H_{12}O_2N_2S$.	Calculated.	C 51.33, H 4.70, N 5.45, S 12.47
	Found.	" 51.81, " 4.93, " 5.65, " 12.57

¹ All melting points recorded herein are uncorrected.

The amount of *p*-fluorophenylmercapturic acid isolated in the above experiment was equivalent to 14 per cent of the *p*-fluorophenyl-*l*-cysteine ingested by the rats.

In an experiment similar to that described above a male white rat weighing 250 gm. consumed 0.200 gm. of *p*-fluorophenyl-*l*-cysteine. The amount of *p*-fluorophenylmercapturic acid, m.p. 158–159°, which was isolated from the urine was 0.035 gm. This corresponded to 15 per cent of the *p*-fluorophenyl-*l*-cysteine ingested by the rat.

SUMMARY

1. *p*-Fluorophenyl-*l*-cysteine has been synthesized and has been converted to *p*-fluorophenylmercapturic acid by acetylation *in vitro*.

2. *p*-Fluorophenylmercapturic acid has been isolated from the urine of rats following the administration of *p*-fluorophenyl-*l*-cysteine in the diet.

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The microanalyses reported herein were performed by Mr. Michael Edson.

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THE METABOLISM OF *dl*-METHIONINE AND *l*-CYSTINE IN DOGS ON A VERY LOW PROTEIN DIET

By LEON L. MILLER*

(From the Department of Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, New York)

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There has been much interest in the beneficial effect of supplementing low protein or non-protein diets with cystine plus choline or with methionine in studies on fatty livers (1), experimental cirrhosis (2, 3), experimental hepatoma (4), and on the detoxication of a variety of organic compounds (5-7). A number of observations have been made of the sulfur and nitrogen metabolism in dogs receiving *single* supplements of cystine (8-10) or methionine (11) with a non-protein diet, but the *repeated daily feeding* of cystine or methionine has not been studied under similar conditions.

The experiments detailed below reveal that the dog, fed a diet containing less than 1 per cent of protein, can utilize extensively the sulfur of *l*-cystine and *dl*-methionine when fed daily over periods of from 8 to 24 days; moreover, when the amino acid supplement is fed to a dog with undepleted protein stores, there is a marked conservation of nitrogen manifested by a sharp decrease in the urinary nitrogen output. This effect becomes less striking as the stores of bodily protein are depleted by continued maintenance on the experimental diet.

The continued utilization of the sulfur-containing amino acid is more marked with *dl*-methionine than with *l*-cystine. In fact, the total urinary sulfur excreted is consistently less than or approximately equal to the total sulfur of the *dl*-methionine supplement.

Methods

All dogs used in these experiments were active, healthy adults. They were taken from the animal house where they were maintained on a diet of mixed table scraps and placed in metabolism cages where they received only water *ad libitum* for 3 to 6 days. Following this, they received approximately 14 gm. per kilo of body weight of a basal diet throughout the whole course of the experiments. The diet consisted of sucrose 72.2 per cent, Wesson salt mixture (12) 4.6 per cent, calcium phosphate 4.6 per cent, Crisco 14.9 per cent, Mazola oil 6.5 per cent, cod liver oil 1.4 per cent, yeast powder (Fleischmann's type 200-B) 0.7 per cent, and powdered liver extract (Lilly, H8083) 0.7 per cent. As indicated in Tables I and II, the

* Lilly Fellow in Pathology and Pharmacology.

Wesson salt mixture was later made "sulfate-free" by replacing the magnesium sulfate with an equal weight of magnesium carbonate. To the daily diet of each dog were added 25 mg. of nicotinic acid, and, unless otherwise stated, 400 mg. of choline hydrochloride. Following preliminary basal periods, *l*-cystine (Eastman Kodak Company) or *dl*-methionine (Merck and Company¹) was fed in 1.000 gm. (267 mg. of sulfur) and 1.250 gm. (268 mg. of sulfur) amounts respectively, as indicated in Tables I and II. A smaller animal, Dog 41-450, received only 0.500 gm. of *l*-cystine per day. The amino acids were thoroughly mixed with the daily diet, and eaten completely over the course of the individual experiments lasting 8 to 24 days.

Levels of plasma protein, which were normal at the start of the experiments, were estimated by macro-Kjeldahl determination on oxalated plasma. As maintenance on the very low protein diet continued, the plasma protein dropped, as indicated in Tables I and II.

The urines were collected daily and preserved with toluene and refrigeration, and, with the exception of Dog 40-402, each period of 48 hours was terminated by catheterization and rinsing of the bladder with water. Total urinary nitrogen was determined by the macro-Kjeldahl method, and urea-ammonia nitrogen by aeration after incubation with urease.

Urinary cystine was determined only on the urine of Dog 40-402, Table II, by using the isolation procedure of Rossouw and Wilken-Jorden (13) and the colorimetric procedure of Lugg (14).

Urinary inorganic sulfate and ethereal sulfate were determined by the procedure of Folin (15). Ethereal sulfate was estimated only on the urine of Dog 40-402, Table II, and, because no significant changes were noted, was not determined in the other experiments.

Total urinary sulfur was determined on an aliquot of the urine by the wet ashing method of Masters (16). The term organic sulfur in Tables I and II refers to the sum of ethereal sulfate sulfur and neutral sulfur. The organic sulfur was obtained by subtracting inorganic sulfate sulfur from total sulfur.

EXPERIMENTAL

Tables I and II contain the data of the experiments carried out without interruption in the dog indicated.

Table I presents data obtained from two dogs in which the addition of the *dl*-methionine was started under different conditions. In Dog 41-418 *dl*-methionine was fed early in the course of the non-protein basal diet, and here the decrease in the total nitrogen and the urea-ammonia fraction is striking in Periods 6 to 15. In Dog 41-542 the feeding of *dl*-methionine

¹ We are indebted to Merck and Company, Inc., for valuable amino acids.

was started after more than 4 weeks on the non-protein basal diet. The dog is hypoproteinemic (with a plasma protein level of 5.0 gm. per cent), and

TABLE I

Metabolism of dl-Methionine in Dog on Very Low Protein Diet

1.250 gm. of *dl*-methionine (268 mg. of sulfur) were fed daily. "Sulfate-free" salt mixture was used throughout in both experiments.

Diet	Period No. (48 hrs.)	Total N	Urea + NH ₄ -N	Un-determined N	Urinary sulfur			Plasma protein
					Inor-ganic	Organic	Total	

Dog 41-418; weight 14.4 kilos									
Basal + choline	1	3.13	2.47	79.1	0.66	158	63	221	Normal
	2	3.29	2.57	78.1	0.72	128	64	192	
	3	2.50	1.84	73.7	0.66	96	54	150	
	4	3.24	2.40	74.2	0.84	103	56	159	
	5	2.49	1.71	68.5	0.78	129	73	202	
Basal + choline + <i>dl</i> -methionine	6	2.31	1.57	68.1	0.74	121	94	215	4.70
	7	1.62	0.80	49.2	0.84	360	126	486	
	8	1.61	0.81	50.5	0.80	415	114	529	
	9	1.62	0.82	51.0	0.80	374	118	492	
	10	1.47	0.65	44.4	0.82	330	129	459	5.20
	11	1.45	0.64	44.5	0.81	349	141	490	
	12	1.60	0.74	46.3	0.86	371	144	515	
	13	1.52	0.72	47.7	0.80	378	133	511	4.95
	14	1.51	0.69	45.8	0.82	339	169	508	
	15	1.50	0.74	49.1	0.76	347	154	501	
Basal + choline	16	1.52	0.75	49.2	0.77	264	194	458	5.36
	17	1.05	0.43	40.5	0.62	143	87	230	
	18	1.21	0.54	44.2	0.67	91	68	159	
	19	1.23	0.55	44.8	0.68	116	54	170	
	20	1.30	0.61	46.7	0.69	78	55	133	

Dog 41-542; weight 12.3 kilos									
Basal + choline	1	1.27	0.72	56.4	0.55	51	23	74	5.00
	2	1.22	0.64	52.5	0.58	70	32	102	
Basal + choline + <i>dl</i> -methionine	3	1.05	0.59	56.1	0.46	147	36	183	4.63
	4	1.12	0.59	53.0	0.53	266	60	326	
	5	1.22	0.60	49.1	0.62	254	118	372	
	6	1.14	0.56	49.1	0.58	237	62	299	
	7	1.17	0.61	52.5	0.55	215	121	336	
	8	1.15	0.55	47.6	0.60	256	144	400	
	9	1.17	0.59	50.6	0.58	252	142	394	
Basal + choline	10	1.15	0.61	53.0	0.54	222	156	378	4.63
	11	1.07	0.54	50.6	0.53	103	113	216	

the effect on the total nitrogen output is definite but small when compared with that seen in Dog 41-418.

In both dogs the output of total urinary sulfur is *less than the methionine sulfur fed*; however, the total sulfur output in Dog 41-418 is apparently approaching a level of "sulfur equilibrium." In Dog 41-542 sulfur retention is greater and the total sulfur output increases more gradually than in Dog 41-418, probably because the sulfur stores of the former are more depleted.

In both dogs the organic sulfur output increases to a point where 18 to 20 per cent of the fed sulfur appears in this fraction.

Table II represents a condensation of data similar to that presented in detail in Table I. The period numbers refer to the 48 hour periods of the actual experiments; however, for economy of space, only the totals for a given supplement and the average 24 hour excretions are indicated. This does *not* reveal the transition of some of the initial periods when the diet supplement is changed, but the conclusions one may draw from these averages are essentially identical with those drawn from the more detailed data.

Table II contains data obtained from Dog 40-402, and it is at once apparent that the addition of *l*-cystine (Periods 5 to 11) to the basal diet plus choline results in a sharp and marked decrease in the average total urinary nitrogen, mainly at the expense of the urea-ammonia nitrogen fraction. There is also marked retention of ingested cystine sulfur.

With the addition of *dl*-methionine (Periods 12 to 18) the excretion of nitrogen continues at the previous low level. There is some increase in the total sulfur excreted during these periods, but definite sulfur retention continues. The increase in organic sulfur in this and the other methionine feeding experiments is probably not attributable to increased output of urinary cystine or ethereal sulfate, since neither of these fractions (which were determined only for the experiments on Dog 40-402) showed any significant increase. Data on the urinary cystine and ethereal sulfate are not recorded in Table II.

Although it is not apparent from Table II, when the basal diet without choline is fed (Periods 19 to 23) it is about 4 days before the total sulfur excretion reaches a basal level again.

The addition of *l*-cystine (without choline) (Periods 24 to 27) at this point, with a plasma protein of 4.53 gm. per cent, causes a slight average fall in the total nitrogen output as compared with the initial effect. Yet the urine became dark and the plasma icteric. The restoration of choline to the diet caused the jaundice to clear and the urine to become light in color; there was no significant effect on the total urinary nitrogen, and there was continued retention of the cystine sulfur, although the output of total sulfur increased somewhat.

TABLE II

Metabolism of l-Cystine, Choline, and dl-Methionine in Dog on Very Low Protein Diet

1.000 gm. of *l*-cystine (267 mg. of sulfur) or 1.250 gm. of *dl*-methionine (268 mg. of sulfur) were fed daily, except to Dog 41-450 which received 0.500 gm. of *l*-cystine (134 mg. of sulfur) daily.

The figures in parentheses represent the average 24 hour excretion.

Basal diet supplement	Period No. (48 hrs.)	Total N	Urea + NH ₃ -N	Un- deter- mined N	Urinary sulfur			Plasma protein	
					Inor- ganic	Organic	Total		
Dog 40-402; weight 12.0 kilos									
Choline	1-4	10.29 (1.29)	7.04 (0.88)	68.4	3.25 (0.41)	873 (109)	252 (32)	1125 (141)	Normal
Choline + <i>l</i> -cystine	5-11	10.40 (0.74)	5.87 (0.42)	56.4	4.53 (0.32)	2893 (207)	516 (37)	3409 (244)	
<i>dl</i> -Methionine	12-18	9.95 (0.71)	5.09 (0.36)	51.2	4.86 (0.35)	3245 (232)	1292 (92)	4537 (324)	
None	19-23	7.98 (0.80)	4.00 (0.40)	50.2	3.98 (0.40)			1662 (166)	4.53
<i>l</i> -Cystine	24-27	6.02 (0.75)	3.21 (0.40)	53.4	2.81 (0.35)	1724 (216)	514 (64)	2238 (280)	
Choline + <i>l</i> -cystine	28-32	7.03 (0.70)	4.00 (0.40)	56.9	3.03 (0.30)	2501 (250)	786 (79)	3287 (329)	
Dog 41-542; weight 12.4 kilos									
Choline	1-2	8.09 (2.02)	6.09 (1.52)	75.3	2.00 (0.50)	423 (106)	219 (55)	642 (161)	6.24
“ + <i>l</i> -cystine	3-9	16.89 (1.21)	10.56 (0.75)	62.6	6.33 (0.46)	3308 (236)	1225 (88)	4533 (324)	
None	10-14	13.19 (1.32)	9.57 (0.96)	72.6	3.62 (0.36)	1142 (114)	578 (58)	1720 (172)	5.63
“ *	15-17	7.04 (1.17)	5.00 (0.83)	71.0	2.04 (0.34)	278 (46)	207 (35)	485 (81)	
Choline	18-21	7.77 (0.97)	5.19 (0.65)	66.8	2.58 (0.32)	352 (44)	271 (35)	623 (79)	5.39
“ + <i>l</i> -cystine	22-26	8.71 (0.87)	5.62 (0.56)	64.5	3.09 (0.31)	1674 (167)	624 (62)	2298 (229)	
Choline + <i>dl</i> -methionine	27-30	6.85 (0.86)	3.86 (0.48)	56.4	2.99 (0.38)	1220 (153)	1063 (133)	2283 (286)	
Choline	31-33	4.35 (0.73)	2.66 (0.44)	61.2	1.69 (0.29)	465 (78)	341 (57)	806 (135)	
Dog 41-450; weight 7.8 kilos									
Choline	1-2	2.95 (0.74)	2.00 (0.50)	67.8	0.95 (0.24)	175 (44)	113 (28)	288 (72)	5.70
“ + <i>l</i> -cystine	3-11	9.18 (0.51)	4.16 (0.23)	45.3	5.02 (0.28)	1936 (108)	752 (42)	2688 (150)	
Choline	12-15	4.10 (0.51)	1.74 (0.22)	42.4	2.36 (0.29)	263 (33)	251 (31)	514 (64)	5.10†

* All subsequent experiments in this table were carried out with “sulfate-free” salt mixture.

† At the start of Period 14.

Table II also contains data from Dog 41-542, and here again (Periods 3 to 9) the protein-sparing action of *l*-cystine is striking, but, in contrast to Dog 40-402 (Periods 5 to 11), there is a considerable increase in the organic sulfur fraction. Since urinary cystine determinations were not done in this or subsequent experiments, the exact nature of this increase is not known. Return to the basal diet *without choline* results in an increased total nitrogen output, mainly in the urea-ammonia fraction. At this point (Period 15) the sulfate-free salt mixture was started and the total sulfur output was almost halved. The restoration of choline to the basal diet (Periods 18 to 21) results in a definite decrease in total nitrogen output with a corresponding fall in the urea-ammonia fraction. The addition of *l*-cystine (Periods 22 to 26) results in a further average marked decrease in the total nitrogen. In Periods 22 to 26 *less total sulfur is excreted than was ingested*. The change from an *l*-cystine to a *dl*-methionine supplement (Periods 27 to 30) results in a small rise in the undetermined nitrogen, associated with a marked rise in the organic sulfur fraction, similar to that seen in Table I. With a return to the basal diet the total nitrogen and undetermined nitrogen decrease by an amount which is approximately equal to that of the fed amino acid nitrogen (0.12 gm. of N per day).

Finally, the data from Dog 41-450 again demonstrate the remarkable protein-sparing action of *l*-cystine (plus choline), with a precipitate fall in the total nitrogen and the corresponding decrease in the urea-ammonia fraction. As compared with most of the *dl*-methionine experiments, the retention of *l*-cystine sulfur is not so marked; assuming a basal total sulfur output of 72 mg. per day, there is a retention of about 900 mg. of sulfur during the feeding periods, Nos. 3 to 11, and in the first follow-up basal period, No. 12, which is not detailed in Table II.

DISCUSSION

The above experiments show that *l*-cystine and *dl*-methionine have a definite protein-sparing action when fed to dogs on a very low protein diet. The extent of the protein-sparing action varies with the extent of protein depletion. When the protein reserve stores are optimal, there is a striking conservation of nitrogen; when the reserve stores have been depleted and hypoproteinemia exists, the nitrogen-sparing action of the sulfur-containing amino acids is less marked. Furthermore, this protein-sparing action becomes maximally apparent only after 2 to 4 days on the amino acid supplement, a fact which may explain Stekol's failure to observe this in his *single feedings* of *l*-cystine (9) and *dl*-methionine (11). In a subsequent study of mercapturic acid synthesis (17) in the dog on a protein-free diet, Stekol fed *l*-cystine for 3 consecutive days and *dl*-methionine for 2 consecutive days; these feedings were started 4 days after the ingestion of bromo-

benzene, and resulted in a marked fall in urinary nitrogen. This was interpreted (17) as showing "that *l*-cystine and *dl*-methionine arrest the breakdown of tissue . . . caused by bromobenzene feeding *after the synthesis and excretion of p-bromophenylmercapturic acid have been completed.*" It seems reasonable that much of this fall of urinary nitrogen is identical with the protein-sparing effect of *l*-cystine and *dl*-methionine seen above in our experiments. Nielsen, Gerber, and Corley (8), however, fed a *much larger single supplement of l-cystine* (5.6 gm.) and noted not only retention of the cystine nitrogen but also a decrease in the output of urinary nitrogen during and after the cystine feeding period.

What is the mechanism of this protein-sparing action? It is conceivable that during periods of protein starvation the organism sacrifices considerable reserve store nitrogen in order to obtain the sulfur-containing and other amino acids essential for its minimal metabolic requirements and for the synthesis of substances richer in cystine or methionine. In the event that the body requires increased amounts of cystine or methionine to detoxicate organic compounds, it is forced to break down body proteins (17) or to restrict its growth (6, 7), unless increased amounts of the amino acids are supplied. Experiments recently reported (18) from this laboratory showed that the addition of only *l*-cystine to a non-protein diet allowed the formation of considerable amounts of plasma protein at the expense of reserve stores already extensively depleted.

The sulfur metabolism studies of our experiments indicate that a variable but large proportion of the sulfur requirement in the dog on a very low protein diet can be provided by a small daily supplement of *l*-cystine or *dl*-methionine for as long as 24 days. *dl*-Methionine is more effective in this respect than *l*-cystine. In many of the experiments the excretion of total urinary sulfur was less than or approximately equal to the sulfur of the cystine or methionine supplement. Because analyses of fecal sulfur were not performed, it can only be assumed that absorption of the amino acids was quantitatively complete, and that positive sulfur balance or sulfur equilibrium existed.

These observations may be of fundamental importance in explaining the action of small supplements of methionine or cystine plus choline in preventing experimental cirrhosis (2, 3), in lowering the incidence of experimental hepatoma (4), and in preventing increased susceptibility to liver poisons (5-7). In a previous report (19) we have presented evidence that in the dog with protein stores depleted by protein deprivation the liver is even more markedly depleted of its sulfur stores.

This has led us to the opinion that the effects of protein depletion in general and sulfur depletion in particular may be interpreted in terms of decreased amounts of sulfur-containing enzymes or lowered activity (or

both) resulting from a decrease in essential sulfhydryl activators such as glutathione. This is in accord with Englehardt's view (20) that cell proteins are primarily enzymes and the recent demonstration (21) that in many of the oxidative enzyme systems involved in carbohydrate metabolism free sulfhydryl groups are associated with full activation. Thus, a deficiency in enzyme systems produced by protein depletion, or inactivation by toxic agents, especially when functional capacity is already impaired by protein depletion, may be the basis of the increased susceptibility to liver damage produced by protein depletion with a diet adequate in other essential factors.

Compared with the very low protein diet used in our experiments, many of the experiments reported in the literature on experimental cirrhosis, fatty livers, etc., have been carried out with diets containing at least 5 per cent protein, usually casein. The protein-sparing action of cystine and methionine probably would be even more marked in such diets, in that nitrogen balances probably would be more favorable. Lewis (22) has demonstrated just such an effect when a diet containing casein and beef heart was supplemented with small amounts of *l*-cystine; *dl*-methionine has been shown to be equally effective (17, 23).

The large increase in organic sulfur of the urine which was consistently noted above when *dl*-methionine was fed, and which was also seen in some of the *l*-cystine experiments, is of interest, since the ethereal sulfur and urinary cystine sulfur accounted for only an insignificant amount of the increase. Stekol noted an increase in neutral sulfur in only one of his methionine single feeding experiments (11). Virtue and Lewis (24) have described a similar increase while administering *dl*-methionine to rabbits on an adequate diet. Since in the case of *dl*-methionine feeding the extra urinary organic sulfur amounted to as much as 30 to 40 per cent of the methionine sulfur fed, it should be possible to isolate this material from the dog's urine.

In one experiment on Dog 41-542, the withdrawal of choline from the basal diet led to an increase in the output of total urinary nitrogen. This effect was reversed by the restoration of choline and suggests that in the absence of an adequate dietary source of labile methyl groups the organism will sacrifice body protein in attempting to meet its minimal requirement.

SUMMARY

1. In dogs maintained on a very low protein diet, both *dl*-methionine and *l*-cystine have a marked protein-sparing action which becomes less striking as reserve stores of protein become depleted.

2. A small daily supplement of *dl*-methionine or *l*-cystine can provide a variable but large proportion of the sulfur requirement in dogs on a very

low protein diet for as long a period as 24 days. *dl*-Methionine is more effective in this respect than *l*-cystine. In fact, the total urinary sulfur excretion with *dl*-methionine feeding does not exceed the total sulfur of the amino acid supplement.

3. In some of the *l*-cystine experiments and in all of the *dl*-methionine experiments, there was a large increase in the urinary organic sulfur fraction equivalent to 15 to 40 per cent of the fed amino acid sulfur. The exact nature of this organic sulfur fraction of urine is not yet known.

4. In a single experiment the addition of choline to the basal diet very low in protein had a definite, though small, protein-sparing action.

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AN IRON-PROTEIN COMPLEX OBTAINED FROM LIVER

BY B. LIBET AND K. A. C. ELLIOTT

(From the Institute of the Pennsylvania Hospital, Philadelphia)

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A protein called "ferritin" which contains 20 to 24 per cent of iron was described by Laufberger (1) and Kuhn *et al.* (2) and has recently been closely studied by Granick *et al.* (3, 4). This material, which crystallizes readily in the presence of cadmium sulfate, is usually prepared from horse spleen, though it is obtainable, in smaller amounts, from other tissues. Preparations containing a different iron-protein complex have now been obtained, by a very simple procedure, from livers of all animals tested (rat, rabbit, guinea pig, pig, beef) but not in appreciable amounts from spleen or brain. We have no evidence to show that the iron-protein complex in our preparations is a single and specific chemical compound but for convenience the material will be referred to as "ferrin." In the accompanying paper (5) it is shown that ferrin behaves in the same way as iron salts in the catalysis of phospholipid oxidation. In the study of that catalysis, ferrin has the advantage over iron salts in being soluble in neutral solution. Possibly ferrin could take the place of iron salts in other iron-catalyzed reactions.

Fresh liver was homogenized with 2 volumes of water, by use of a mechanical mixer, and the suspension was heated in a water bath to 90°, strained through gauze, and the liquid filtered. Sufficient 3 N HCl was added to bring the pH to about 2.5 and the precipitate which formed was centrifuged down, washed with water, and dried *in vacuo*. From 1 kilo of pig liver, 2.2 gm. of a reddish brown acid precipitate were obtained which contained 6.3 per cent of iron.¹ (Adjusting the pH of the liver extract to 4.6, instead of 2.5, gave a smaller yield of precipitate, 1.1 gm. per kilo, with a slightly higher iron content, 8.1 per cent.)

Partial purification of the iron-protein was obtained as follows: 500 mg. of the acid precipitate were dissolved in 25 cc. of water, sufficient dilute NaOH being added to give a clear neutral solution. To this, 1.7 gm. of ammonium sulfate were added, and the pH brought back to 7.1 with drops of NaOH solution. The precipitate which formed was centrifuged down

¹ Iron was determined by the method of Granick (3) except that the digestion was done by heating for an hour with 1.5 cc. of 70 per cent perchloric acid, then adding 0.5 cc. of 30 per cent hydrogen peroxide, and heating a further 20 minutes. Sharp end-points were always obtained in the subsequent permanganate titration, whereas very indefinite end-points were obtained after digestion with sulfuric and nitric acids.

and redissolved in 25 cc. of water with a little NaOH. The solution was dialyzed for 4 days against running distilled water. The ferrin precipitated toward the end of the dialysis, probably as a result of the slight acidity of the distilled water (pH about 5.5). The supernatant fluid was centrifuged off and the precipitate dried *in vacuo*. 141 mg. of material containing 15.7 per cent of iron were obtained.

The acid precipitate, or purified ferrin, is almost insoluble in water but dissolves readily on the addition of sufficient alkali to bring the pH to 8 or 9 and remains in solution when the pH is brought back as low as 6.7. The material gives positive biuret, xanthoproteic, Hopkins-Cole, and Milon tests for protein. Strong solutions are colored a deep brown; dilute solutions, 0.5 mg. per cc., are brownish yellow. Viewed through a hand spectroscope, the solutions show no absorption bands, and treatment with alkali, pyridine, and hydrosulfite produces no hemochromogen bands. Treatment with 0.3 N HCl and ferrocyanide or thiocyanate gives a strong reaction for iron. The iron appears to be in the ferric state, since potassium ferricyanide or α, α' -bipyridine and dilute acid, air being excluded, give no color; only after reduction with hydrosulfite does the test become positive. When ferrin is treated with strong HCl, a yellow solution is obtained; if this solution is gradually neutralized and made alkaline, protein precipitates out and then redissolves, leaving a precipitate of ferric hydroxide. Evidently treatment with strong acid irreversibly destroys the ability of the protein to combine with iron. Ferrin may be dissolved in strong NaOH solution without suffering any apparent change.

Ferrin resembles ferritin in being a protein containing large amounts of iron which can be separated from the protein by treatment with acid but not by dialysis from neutral solution. It differs from ferritin in several respects. On addition of cadmium sulfate to ferrin solutions, no crystals were formed but merely a large amorphous precipitate. Ferrin was precipitated by a lower concentration of ammonium sulfate than is ferritin. Little or no acid precipitate was formed from horse spleen extract, heated to 80°, which is the richest source of ferritin. As is shown in the accompanying paper, the iron in ferrin is more active than the iron in ferritin in the catalysis of phospholipid oxidation.

Since heat is involved in its preparation, it is possible that ferrin is a denatured product. Attempts to obtain preparations by methods not involving heat coagulation of other tissue proteins have not yet been successful. Whether or not ferrin is a denatured product, it is a substance of interest in view of its high, catalytically active, iron content and as a form in which large amounts of iron may be held in neutral or alkaline solution when ionizable iron is almost completely precipitated. Brückmann and Zondek (6) found the content of non-hemin iron of dry human, rat, and

calf livers, to be about 98, 69, and 35 mg. per 100 gm. of dry tissue respectively. The yield of iron in the acid precipitate was about 48 mg. per 100 gm. of dry pig liver. It seems probable that much of the non-hemin iron in liver is normally present in some form of combination with protein either as ferrin or as a substance which is changed to ferrin by heat.

SUMMARY

An iron-protein complex, soluble in neutral or alkaline solution, has been obtained from liver. Partially purified, the material contains 15.7 per cent of iron.

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OXIDATION OF PHOSPHOLIPID CATALYZED BY IRON COMPOUNDS WITH ASCORBIC ACID

BY K. A. C. ELLIOTT AND B. LIBET

(From the Institute of the Pennsylvania Hospital, Philadelphia)

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Thunberg (1) found that traces of iron salts accelerated the oxygen uptake of emulsions of phospholipid preparations. Warburg (2) found that iron salts stimulate the respiration of "granular suspensions" of sea urchin eggs and promote oxygen uptake by commercial egg lecithin and linolenic acid. Maximum rates were obtained in weakly acid solutions. More recently Panimon, Horwitt, and Gerard (3) have shown that ferric and ferrous salts increase the rate of oxygen uptake of suspensions of brain and other tissues and they concluded that phospholipids, and also proteins, were oxidized. Deutsch, Kline, and Rusch (4) have found that, in the presence of ascorbic acid at pH 4, liver phospholipid takes up oxygen at a considerable rate.

We have confirmed the findings of Panimon *et al.* and Deutsch *et al.* but have found that ascorbic acid and very small amounts of iron together cause a very great acceleration of the oxygen uptake of brain and liver suspensions and of phospholipid at neutral pH. The kinetics of the oxidation of the iron-ascorbic acid-phospholipid system are peculiar and the system is affected by several substances of physiological interest.

For many of the experiments reported, the iron added was in the form of the iron-protein complex obtained from liver which was described in the preceding paper (5) and is referred to as ferrin. Ferrin iron behaved in general in the same way as iron salts but had the advantage of being soluble in neutral solution.

Methods

Oxygen uptake was measured with Barcroft differential manometers at 38°. The gas space contained air and the total volume of liquid was 3 cc. Neutralized ascorbic acid and iron solutions were added from side bulbs. Since the oxidation of ascorbic acid is strongly catalyzed by traces of copper (6), all solutions were made up in, and glassware rinsed with, water which had been twice redistilled in all-glass apparatus. For most experiments this precaution may not have been necessary, since both ferrin and the phospholipid strongly inhibited the catalytic effect of added copper salt on ascorbic acid oxidation.

Tissues and lipids were homogenized, by means of the apparatus of Potter

and Elvehjem (7), in sodium phosphate buffer, M/60 for tissues, M/30 for lipids, usually at pH 7.0. Fatty acids were reduced to quite stable emulsions by adding buffer and about an equivalent of NaOH solution and then sufficient HCl solution to return the pH to 7.0. Iron salts, solid ferrous ammonium sulfate and a standard solution of ferric chloride, were added to large volumes of buffer solution and the pH adjusted with drops of 0.5 N NaOH. In this way iron salt suspensions which settled slowly were obtained. All suspensions were freshly prepared for each experiment.

For experiments with the iron-protein complex, the crude "acid precipitate" described in the preceding paper, containing 6.3 per cent of iron, was used. This was dissolved in phosphate buffer with the aid of a little NaOH and the pH was then adjusted with HCl.

Effects of Ascorbic Acid and Iron on Tissue Suspensions—As is shown in Fig. 1, the addition of ascorbic acid to hypotonic brain suspension caused the initial respiration rate to be maintained better. The addition of ascorbic acid (0.001 M) and iron (5 micromoles in 3 cc.) produced a very large acceleration of the initial rate of respiration, though the effect fell off rapidly. Iron alone caused a smaller but appreciable initial stimulation. The effect of iron alone probably depends on the ascorbic acid present in the tissue. Bessey and King (8) found about 0.36 mg. of ascorbic acid per gm. of rat brain, which would be sufficient to produce the observed activity of added iron. With well washed brain suspension, the effect of iron without ascorbic acid practically disappeared but became fully apparent on addition of ascorbic acid. Rat liver suspensions behaved very similarly to brain suspensions.

Brain tissue suspensions which had been heated to 100° for 15 minutes behaved similarly to washed suspensions. Acetone-dried brain retained its activity but, after exhaustive extraction with benzene or alcohol and ether, iron and ascorbic acid had little or no effect, indicating the lipid nature of the oxidizable material.

With brain suspensions prepared in isotonic medium the respiration rate is much greater than with hypotonic suspensions (9) and added ascorbic acid alone had almost no effect. When iron and ascorbic acid were added, the usual large initial acceleration of oxygen uptake occurred, but after about 90 minutes the rate fell off to well below the control value (Fig. 2). Evidently the iron-catalyzed oxidation results in destruction of structures or compounds upon which the normal respiration depends.

The effects of ferrous and ferric iron and the iron-protein complex were similar. The extra oxygen uptake caused by iron compounds was not accompanied by increased CO₂ evolution.

Mixed Brain Phospholipid—Most of this work has been done on purified mixed phospholipids prepared from beef brain according to Chargaff *et al.*

(10). The material, when freshly prepared, was almost pure white. It was stored in evacuated bottles in the refrigerator and any which became markedly yellow, as a result of repeated exposure to air, was discarded. The oxygen uptake by emulsions of this material without additions was almost imperceptible. Addition of ascorbic acid alone caused an appreciable oxygen uptake. Addition of small amounts of iron alone had no effect. But iron and ascorbic acid together caused a very rapid initial oxygen uptake. The rate fell off steadily, tending toward a total uptake which depended upon the concentrations of the components of the system. No CO_2 was evolved.

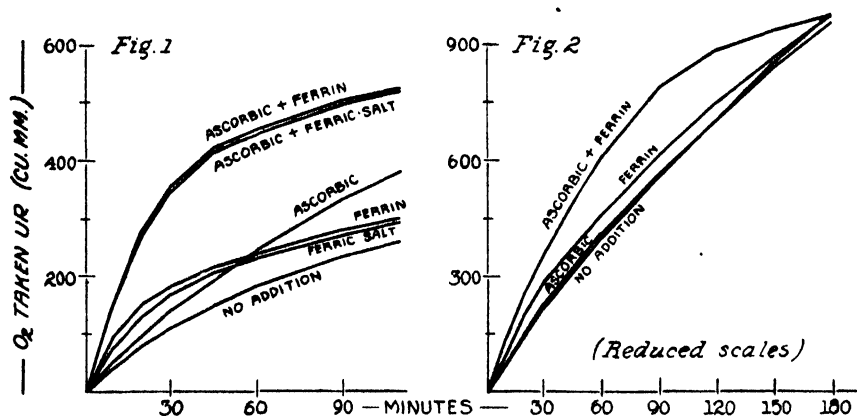


FIG. 1. Effects of iron and ascorbic acid on hypotonic brain suspension. Fresh weight of tissue, 300 mg.; ascorbic acid, 0.001 M; ferric salt, 5 micromoles (0.0017 M); crude ferrin, 5 mg. (0.0018 M ferrin iron); pH 7.0.

FIG. 2. Effects of iron and ascorbic acid on isotonic brain suspension containing glucose. Same additions as in Fig. 1.

In phosphate buffer solution the activity of the system increased considerably with increasing hydrogen ion concentration. The curves shown in Fig. 3 were obtained with iron added as ferrin which was completely in solution except at pH 6. In acetate buffer at pH 4.7, ascorbic acid, 0.003 M, alone gave a more rapid oxygen uptake than at pH 7, and ferric salt (1 micromole of suspension in 3 cc.) gave a very slow uptake. Ferrous salt (1 micromole of suspension in 3 cc.) gave an oxygen uptake which was not affected by the presence or absence of ascorbic acid; the uptake continued for a long time but followed an erratic course and the initial rate was actually lower than with ascorbic acid alone. The erratic course of the uptake and the inhibition of the effect of ascorbic acid are probably connected with the formation of a clumpy precipitate some time after the iron suspension and phospholipid emulsion are mixed. In 0.1 N HCl at pH 1.3, the

phospholipid was largely precipitated and the effect of ascorbic acid alone was decreased again. Ferrous salt, with or without ascorbic acid, caused a rapid but still erratic oxygen uptake.

It seems that ferrous iron is the active catalyst. At pH 7 or higher, ferrous salt alone is quickly oxidized but the presence of ascorbic acid probably keeps the iron in the reduced, active state. At acid pH values, ferrous iron is not readily oxidized by oxygen, so that ascorbic acid is not needed; ferric iron, however, has to be reduced before it becomes active.

With constant amounts of ascorbic acid and iron, the rate, and total amount, of oxygen uptake increased on increase of the phospholipid concentration from 9 to 60 mg. in 3 cc., though not quite proportionately. The initial rate was increased about 15 per cent by substituting oxygen for air.

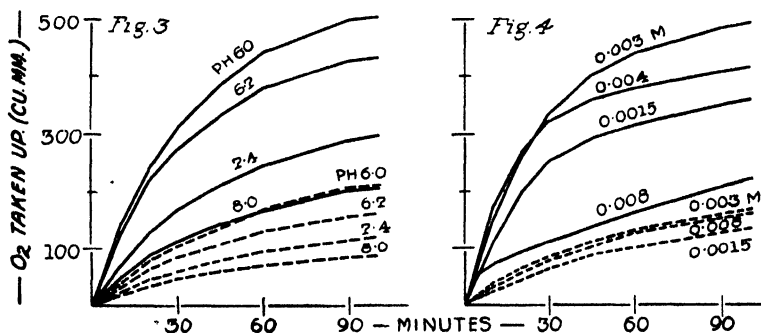


FIG. 3. Effect of pH. Phospholipid, 30 mg. Continuous lines, experiments with ascorbic acid, 0.002 M, plus crude ferrin, 0.3 mg. (1.1×10^{-4} M ferrin iron); broken lines, with ascorbic acid alone. The solutions were adjusted to the pH values shown, with a glass electrode.

FIG. 4. Effects of ascorbic acid concentration. Phospholipid 30 mg.; ascorbic acid concentrations as shown. Continuous lines, with 5 mg. of crude ferrin (0.0018 M ferrin iron); broken lines, no added iron. pH 7.0.

Increasing low concentrations of ascorbic acid increased the initial rate and total amount of oxygen uptake towards a maximum but further increase in ascorbic acid concentration resulted in inhibition or early falling off of the rate (Fig. 4). A lower concentration of ascorbic acid was required to produce inhibition if the iron concentration was raised. With one preparation of phospholipid, the optimum ascorbic acid concentration was about 0.004 M in the presence of 0.3 mg. of crude ferrin in 3 cc. (10^{-4} M ferrin iron) and about 0.003 M with 5 mg. of crude ferrin in 3 cc. With another preparation the optimum ascorbic acid concentration was lower, 0.0015 M with 5 mg. of ferrin.

The initial rate and the total amount of oxygen uptake increased with increasing low iron concentrations towards a maximum at about 1 micro-

mole of iron in 3 cc. (Fig. 5), and with amounts above 5 micromoles the rate fell off early, just as with high concentrations of ascorbic acid. At all concentrations less than the optimal, approximately half the total oxygen uptake occurred in the first 30 minutes.

The tendency to reach a maximum oxygen uptake cannot be ascribed to exhaustion of phospholipid, since different maxima were reached with different amounts of iron. Nor can it be accounted for by destruction of ascorbic acid, since excess of this induced earlier inhibition. It seems likely that an inhibitor must be produced by the reaction and accumulate with time.

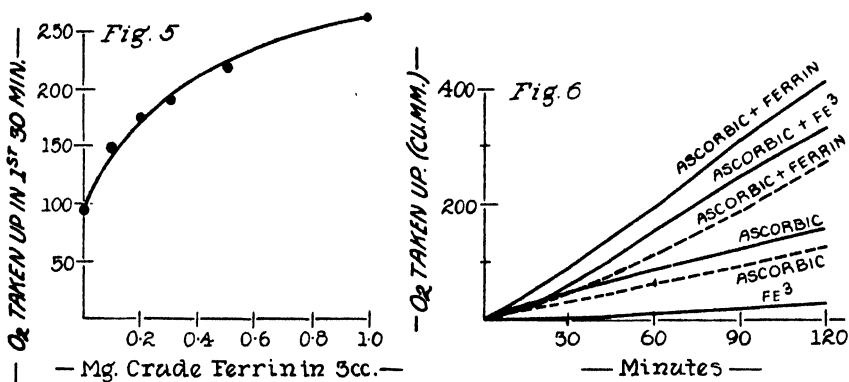


FIG. 5. Effects of iron concentration. Phospholipid 30 mg., ascorbic acid, 0.003 M; pH 7.0. (1 mg. of crude ferrin in 3 cc. makes 3.7×10^{-4} M ferrin iron.)

FIG. 6. Effects of iron and ascorbic acid on unsaturated fatty acids. Continuous lines, linolenic acid, 40 mg.; broken lines, linoleic acid, 40 mg. Ascorbic acid, 0.003 M; ferric salt, 1 micromole (3.3×10^{-4} M Fe); crude ferrin, 1 mg. (3.7×10^{-4} M ferrin iron); pH 7.0.

With brain tissue suspensions, similar inhibitory effects of excess ascorbic acid (above 0.003 M) and iron (above 10 mg. of ferrin in 3 cc.) were observed.

Other Lipid Preparations—Mixed phospholipid was prepared from pig liver by the same method as the brain phospholipid; it was initially quite yellow. With ascorbic acid alone at pH 7, it took up oxygen much more rapidly than did brain phospholipid but the rate and amount of oxygen uptake were still increased by the further addition of iron.

Of the brain phospholipids, cephalin was the only fraction which behaved like the mixed phospholipid preparation. Cephalin was prepared from beef brain according to the method of Folch and Schneider (11). Beef brain lecithin, prepared through the cadmium compound by the procedure given in Hawk and Bergeim (12), was quite inactive. The inactivity was not due to inhibition by traces of cadmium, since less than

10^{-6} M cadmium could be detected in the emulsion by the dinitrophenyl-carbazide test (13), whereas 10^{-3} M cadmium added gave a strong test but less than 30 per cent inhibition with mixed phospholipid. A preparation which presumably contained sphingomyelin was almost inactive. To obtain this material a concentrated petroleum ether extract from acetone- and alcohol-extracted beef brain was treated with 5 volumes of absolute alcohol and the precipitate was dissolved in ether. On standing in the refrigerator, a precipitate appeared which should consist of sphingomyelin and some cerebroside. The precipitate was washed with ether, alcohol, and ether and dried *in vacuo*.

A preparation of cerebroside was also inactive. To obtain this material, beef brain, after extraction with acetone and petroleum ether, was extracted with hot pyridine. The pyridine extract was poured into 4 volumes of acetone and the white precipitate was crystallized three times from alcohol-chloroform mixture and dried *in vacuo*. Cholesterol, glycerol, and stearic and oleic acids were also inactive.

Linoleic and linolenic acids (both obtained from A. D. Mackay, New York) gave appreciable oxygen uptake with ascorbic acid alone, and on addition of ferrin or iron salts with ascorbic acid the rate increased considerably, but it was not nearly as high as during the initial period with phospholipid (Fig. 6).

Specificity of Ascorbic Acid Activity—Though the activity of ascorbic acid in potentiating the oxygen uptake of phospholipid with iron at neutral pH is presumably connected with its reducing property, we have not been able to obtain any appreciable effect with equivalent amounts of cysteine, glutathione, hydroquinone, epinephrine, or sulfite. Sulfite rapidly took up just the amount of oxygen required to produce sulfate, even in the absence of iron. Cysteine took up rapidly about twice the amount of oxygen required to produce cystine, whether iron was present or not.¹

It seems possible that its action as a cocatalyst of the oxidation of phospholipid material may be a specific activity of ascorbic acid in biological systems.

Activity of Iron in Different Forms—In order to compare, approximately, the activity of iron in different forms, experiments were run with equal samples of the same solutions of phospholipid and ascorbic acid to which were added, in different manometer flasks, 0, 0.1, and 0.5 mg. of a standard ferrin preparation and two different amounts of the unknown which would not exceed the activity of the higher standard. The uptakes of the stand-

¹ In the absence of phospholipid or iron, cysteine oxidation alone is very slow and in the presence of iron alone not more than the theoretical uptake to produce cystine occurs. Evidently phospholipid and cysteine or cystine interact in some unknown way.

ards in 30 minutes were plotted against the amount of standard as in Fig. 5. The amounts of standard corresponding to the uptakes of the unknowns at 30 minutes were read from the standard curve.

Per unit weight of iron, the activities of solutions of different preparations of crude ferrin and of suspensions of inorganic ferric and ferrous salts were about equal. But in general there was no correlation between iron content and activity of different iron-protein preparations except that preparations containing no iron showed no activity. If 100 is taken as the activity per unit weight of iron in crude ferrin (the precipitate at pH 2.5 from heated liver extract), the activities per unit weight of iron in two preparations of purified ferrin, containing 12.4 and 15.7 per cent of iron, were respectively 74 and 50. The preparation containing 15.7 per cent of iron is described in the preceding paper (5); the other preparation was obtained similarly but without ammonium sulfate precipitation. A precipitate, obtained by adjusting heated liver extract to pH 4.6, contained 8.1 per cent of iron and its activity per unit weight of iron was 20. Cadmium-free ferritin, containing 17.6 per cent of iron, and thrice crystallized cadmium ferritin, containing 19.2 per cent of iron, showed activities per unit weight of iron of only 11 and 9. These materials were prepared from horse spleen according to Granick (14); both preparations were thoroughly dialyzed and the solutions were dried from the frozen state. On addition of 30 gm. of ammonium sulfate per 100 cc. to heated liver extract, the iron-protein was all precipitated; saturation of the mother liquor with ammonium sulfate gave a precipitate containing no iron and showing no activity. The precipitate obtained at pH 2.5 from heated brain extract contained practically no iron and showed no activity.

Potassium ferri- and ferrocyanide were inactive. Cytochrome *c* preparations, 0.5 to 1.0 mg. in 3 cc., were inactive, while hemin, 0.3 to 1 mg. (1.5 to 5×10^{-4} M), and nicotine hemochromogen (10^{-4} M hemin plus 0.05 M nicotine neutralized) inhibited the uptake which occurred with phospholipid and ascorbic acid alone. Small amounts of hemoglobin (cytolysed human red cells and dried dog hemoglobin) behaved very much like ferrin or iron salts but were very much more active per unit weight of iron.² But there was a slight lag before the full effect of hemoglobin was evident and, after experiments with small amounts of hemoglobin, spectroscopic examination of vessel fluids treated with alkali, pyridine, and hydrosulfite showed no, or much reduced, hemochromogen bands indicating destruction of the heme nucleus. Further, increasing the amount of hemoglobin in the 3 cc. of emulsion from 0.1 to 0.5 mg. caused a relatively small increase

² Lemberg *et al.* (15) found that oxyhemoglobin with ascorbic acid took up oxygen with the formation of choleglobin and dehydroascorbic acid. This oxygen uptake did not occur appreciably under our conditions.

in uptake rate and 5 mg. had the same effect as 50 mg. It is therefore probable that the actual catalyst was a breakdown product of hemoglobin.

Inhibitors and Accelerators—The oxygen uptake by phospholipid plus ascorbic acid alone was appreciably inhibited by cyanide. But 0.01 M cyanide (neutralized) caused only a slight decrease in the initial oxygen uptake rate of phospholipid plus ascorbic acid plus iron salt or ferrin (Fig. 7), or hemoglobin; the rate was better maintained and a greater total uptake occurred. Cyanide is commonly regarded as a general inhibitor of oxidations catalyzed by iron and the present system is therefore unusual.

The effect of iron and ascorbic acid on tissue suspensions or purified phospholipid was almost completely inhibited by 10^{-3} M epinephrine, less completely by tyramine and 3,4-dihydroxyphenylalanine. Catechol and

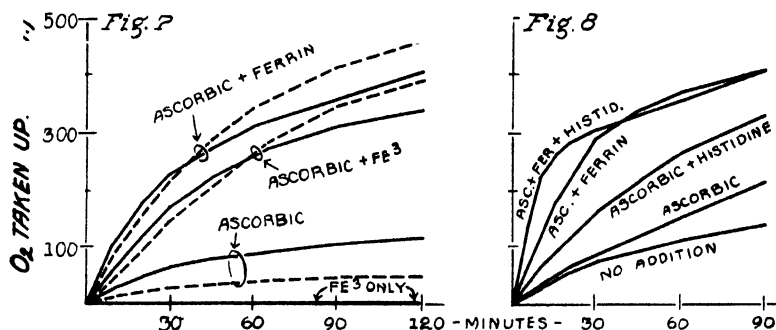


FIG. 7. Effect of cyanide. Phospholipid 30 mg.; ascorbic acid, 0.002 M; ferric salt, 0.24 micromoles (0.8×10^{-4} M); crude ferrin, 0.5 mg. (1.8×10^{-4} M ferrin iron); pH 7.4. Continuous lines, without cyanide; broken lines, with 0.01 M cyanide.

FIG. 8. Effect of histidine with ascorbic acid and iron on brain suspension. Tissue, 200 mg.; ascorbic acid, 0.001 M; crude ferrin, 5 mg. (0.0018 M ferrin iron); $l(-)$ -histidine, 0.01 M; pH 7.0.

hydroquinone were also strongly inhibitory and phenol had an appreciable effect. Phenylpyruvic acid was also strongly inhibitory.

Neutralized horse or rat serum, 1.2 cc. in 3 cc. total, had little effect on the uptake of brain tissue or purified phospholipid with ascorbic acid alone but it almost completely inhibited the effect of added iron. The effect could be largely accounted for as inhibition by calcium. Calcium chloride, 0.0013 M, inhibited the effect of iron about 90 per cent. Magnesium chloride in the same concentration inhibited only about 20 per cent. Soluble protein also inhibited considerably. Gelatin and egg albumin, 10 mg. per cc., inhibited about 25 and 45 per cent respectively.

With brain suspensions, 0.01 M histidine increased the effect of added ascorbic acid and increased still more the initial effect of ascorbic acid plus iron, though the uptake rate fell off earlier (Fig. 8). Similar but less pro-

nounced effects were obtained with a number of other *d*- and *l*-amino acids. With purified phospholipid, histidine increased the effect of ascorbic acid added alone. However, there was no stimulation of the initial effect of ascorbic acid plus iron on purified phospholipid, though the early fall in oxygen uptake was marked.

DISCUSSION

The above work suggests that inorganic iron or soluble iron-protein compounds, together with ascorbic acid, may play a part in the metabolism of lipid material and may also have significant effects on permeability and other functions of nervous tissue and cell surfaces since phospholipids are prominent constituents of these. The sensitivity of the oxidizing system to epinephrine, calcium, and certain amino acids may be a clue to some of the specific actions of these substances. However, it will be difficult to bring the observations into clear relation with other fields of biochemistry until information concerning the products of the oxidation and the mechanism of the interaction of the components of the system is available.

It should be pointed out that heated liver extract is the source of the iron-protein complex referred to as ferrin and, as commonly prepared, would contain at least 1 mg. of ferrin per cc. Addition of such extracts to respiring tissue suspensions produces considerable acceleration of respiration followed by inhibition (9), just as iron does even without added ascorbic acid. It is probable that some of the results of various workers who have added heated tissue extracts as sources of mixed coenzymes may be complicated by the effects of ferrin.*

SUMMARY

1. The respiration of tissue suspensions of brain or liver is stimulated for 30 to 40 minutes by the addition of small amounts of iron or iron-protein complex. This stimulation is greatly increased by the addition of ascorbic acid. Ascorbic acid alone causes the respiration of hypotonic tissue suspensions to be better maintained but has no effect on isotonic suspensions.

2. With purified mixed phospholipid from brain or liver at neutral pH, ascorbic acid causes some oxygen uptake. Ascorbic acid plus iron com-

* In fact, iron impurities in various materials added in experiments on tissue respiration can lead to erroneous conclusions. For instance, stimulation of brain respiration by iron impurity in several glycogen preparations led us (16) previously to report erroneously (17) that glycogen was readily oxidized by brain tissue through a system different from that for glucose. Glycogen may be freed of the impurity by precipitation, once or twice, with alcohol from solution in 1 N hydrochloric acid.

pound causes very rapid initial oxidation, the rate falling off with time. The activity of the system varies in a complex manner with varying concentrations of the components.

3. Of brain phospholipids, only the cephalin fraction undergoes oxidation with iron and ascorbic acid. Linoleic and linolenic acids are oxidized but more slowly than mixed phospholipid or cephalin.

4. No other reducing agent tried was able to replace ascorbic acid in the system.

5. The effects of different iron-protein preparations are not proportional to their iron content. Ferritin is less active than ferrin. Hemoglobin in small amounts is destroyed by phospholipid and ascorbic acid, giving rise to an active catalyst of the oxidation. Cytochrome *c* and hemin are inactive.

6. Cyanide causes little inhibition of, and maintains for a longer time, the effects of iron and ascorbic acid. Epinephrine and other phenols are strongly inhibitory. Serum is strongly inhibitory; its effect may be largely accounted for by inhibitory effects of calcium and of protein. Some amino acids, especially histidine, have marked stimulatory and subsequent inhibitory effects on the system.

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A BLUE CHROMOPROTEIN FOUND IN THE EGGS OF THE GOOSE-BARNACLE

By ERIC G. BALL

(From the Marine Biological Laboratory, Woods Hole, the Department of Physiological Chemistry, the Johns Hopkins University School of Medicine, Baltimore, and the Department of Biological Chemistry, Harvard Medical School, Boston)

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The eggs and embryos of the goose-barnacle¹ contain a pigment the color of which depends upon the age of the developing embryo. Dr. D. P. Costello, who drew my attention to this pigment, furnishes the following description:

"The eggs in the ovary and the earliest cleavage stages in the egg lamella are a distinct pale blue. By the gastrula stage, the color becomes lavender with a blue spot remaining in the yolk interior slightly above the equator of the egg. The early nauplii are somewhat less blue and more pink in color, the greatest intensity of color being in the interior of the nauplius. Hatched nauplii from the mantle cavity, when examined in mass, are entirely pink, having lost the blue color entirely. There is thus a gradual transition from blue to pink during embryonic development."

There will be described in this paper experiments which indicate that the blue pigment of the eggs is a carotenoid-protein compound. Dissociation of this complex into its component parts results in the production of a colorless protein and a red carotenoid-like substance. It would appear that the change in color of the developing embryos must be ascribed to a similar dissociation of the carotenoid-protein complex.

Extraction and Properties—The egg lamella, which consisted of a flat sheet of eggs, was removed from the mantle cavity. Most of those obtained were blue in color. Those that were lavender or pink in color were segregated and worked up separately. The blue eggs were ground in a mortar with sand and water and the mixture centrifuged. The turbid blue supernatant was decanted and the residue further extracted with 1 per cent

¹ Goose-barnacles are not indigenous to the Woods Hole waters. They are occasionally found in this region at various times between June and September when prolonged southeast winds have carried drifting material of the Gulf Stream in this direction. For the most part, those employed in this study were obtained from a floating piece of timber picked up in Vineyard Sound during the summer of 1940. According to Dr. Costello, they were probably *Lepas anatifera*, though positive identification was not made. During the summer of 1943, a few specimens of *Lepas fascicularis* were obtained. The eggs of this species contain a blue pigment with properties similar to those described here. The spectrophotometric data presented were obtained entirely on the pigment from *Lepas fascicularis*.

ammonium sulfate until it was colorless. The pigment was not water-soluble in the absence of salt. The centrifuged extracts were then combined and brought to 50 per cent saturation with ammonium sulfate. The precipitated pigment was centrifuged off and dissolved by the addition of a volume of water equal to the volume of the original extracts. The blue solution so obtained was opalescent and contained some undissolved material. Ammonium sulfate was now added to make a 25 per cent saturated solution and the precipitate centrifuged down and discarded. The supernatant, which was water-clear and deep blue in color, was then brought to 42 per cent saturation with respect to ammonium sulfate. The precipitated pigment was centrifuged off and dissolved by the addition of water. Dialysis of the resulting clear blue solution at 6° overnight against distilled water caused the pigment to precipitate as the salts were removed. The precipitated pigment could be separated by centrifugation and dissolved in weak salt solutions. This step was, however, of little value in purification of the pigment, since a loss in color intensity occurred. This loss in color on dialysis does not appear to be due to the loss of a dialyzable prosthetic group, since solutions of the pigment also lose their color slowly on standing in the refrigerator. At room temperature and in the presence of light and oxygen, solutions of the pigment lost most of their color within 12 hours. No attempts were made to prevent this fading by the use of an inert atmosphere, though it seems likely that it is due to an oxidative reaction. The pigment, when in the precipitated state under ammonium sulfate, may be kept in the refrigerator for weeks without any apparent loss in color. All experiments to be described subsequently were, therefore, performed upon solutions freshly made from a precipitate of the pigment preserved under ammonium sulfate.

Attempts to extract the pigment from the pink or lavender eggs by a similar procedure were unsuccessful. A pigment could be removed, however, if 95 per cent ethyl alcohol or methyl alcohol was employed for extraction purposes. The alcohol solutions so obtained contained a red pigment which was similar in properties to that which could be obtained from the blue eggs and which will be described subsequently.

At first glance, it seemed not unlikely that the difference in color of different batches of eggs could be attributed to a difference in acidity, a change from blue to pink occurring perhaps as development occurred and metabolic processes increased the acidity. Indeed, addition of a strong acid to the blue eggs caused them to turn red. However, the subsequent addition of alkali did not restore the blue color. Moreover, the addition of alkali to the blue eggs also caused them to turn red. It was then found that any process such as the application of heat or the addition of alcohol which brought about the denaturation of the protein caused a change in color from

blue to red. In the case in which alcohol was added, the pigment dissolved to leave a colorless residue. In all other cases, the pigment was precipitated out of solution along with the denatured protein.

This behavior of the pigment suggested that it was similar to the green carotenoid-protein pigment to be found in lobster eggs and shells. Stern and Salomon (3) in studying ovoverdin, the pigment of the lobster egg, showed that if solutions of this pigment were heated rapidly to 65–70° they changed from green to red and, if they were then quickly cooled, the green color returned. This process could be repeated but not indefinitely, since some denaturation of the protein occurs each time. Solutions of the blue pigment from goose-barnacle eggs were found to display similar properties. When heated to 60–65°, the color changed from blue to lavender to pink. If cooled immediately in ice water, the blue color was nearly completely restored, though indications of some irreversible changes were evident. Repetition of the process eventually resulted in complete irreversibility of the reaction and the production of a red precipitate.

This reversible thermal dissociation of the chromoprotein suggested that a similar effect might be obtained by the cautious acidification of the pigment. However, the slow addition of acid to buffered solutions of the pigment until the red form was produced always resulted in denaturation of the protein and the consequent irreversibility of the reaction. There was then tried a procedure first introduced by Warburg and Christian (5) of acidification in the presence of ammonium sulfate in order to protect the protein from denaturation. This proved to be successful. A solution of the pigment was 25 per cent saturated with ammonium sulfate, placed in an ice bath, and ice-cold 1 N HCl added in 0.01 to 0.02 cc. increments, slowly and with swirling of the solution. The solution gradually became turbid and then the color changed from blue to lavender to light red as more acid was added. The addition of acid was stopped as the red color was reached. If cold 0.1 M Na_2HPO_4 was then added, the original blue color was nearly completely restored and only a slight turbidity remained. Centrifugation of the mixture yielded a clear blue solution which had the appearance of the original untreated solution.

The experiment was then repeated on this same solution, but instead of being immediately reneutralized as the red color was again reached, cold saturated ammonium sulfate was added to make the solution 50 per cent saturated with respect to this salt. The red precipitate formed was centrifuged off in the cold and the clear colorless supernatant discarded. Upon addition of 0.1 M Na_2HPO_4 to the red precipitate, it dissolved nearly completely without, however, turning blue. Centrifuging now yielded a clear red solution and no manner of treatment would turn it blue. Heating the solution coagulated the protein and a red precipitate of the pigment and

protein resulted. The possible significance of these findings will be discussed later.

An attempt was next made to obtain some idea of the pH range within which this color change occurred. The blue pigment was therefore dissolved in 0.1 M Na_2HPO_4 and brought to 25 per cent saturation with ammonium sulfate. Four aliquots of the ice-cold mixture were then treated with 1 N HCl so as to produce color changes which by eye were judged to represent 25, 50, 75, and 100 per cent conversion from the blue to the red form. The solutions were then brought to room temperature and their apparent pH values determined with the glass electrode. The pH values obtained were 4.13, 3.61, 3.37, and 3.24. The pH at the point where the color was lavender and the conversion judged to be 50 per cent complete was thus 3.61. If it can be assumed that the color change represents the dissociation of a weak acid, then its pK value must be in the neighborhood of 3.6.

Some of the properties of the colored prosthetic group were next investigated. This could be obtained free from the protein by extracting it with 95 per cent ethyl alcohol from an ammonium sulfate precipitate of the pigment or from the eggs themselves. The clear, orange-red, alcoholic solution was separated from the colorless protein residue by centrifuging. Evaporation of the alcohol at room temperature left a water-insoluble oily red residue. This residue dissolved readily in ethyl ether and when such a solution was underlaid with concentrated H_2SO_4 , a faint green color could be observed at the interface of the two liquids. The residue was also soluble in petroleum ether and the pigment could be extracted completely from this solvent with 90 per cent methyl alcohol. Solutions of the pigment when viewed with a hand spectroscope showed a broad absorption band in the region of λ 500 m μ . These properties of the pigment are characteristic of carotenoids of the xanthophyll type (*cf.* Zechmeister (6)).

Kuhn and Sørensen (1) have described the isolation of a red carotenoid pigment which they have called astaxanthin from the green chromoprotein found in lobster eggs. These workers have reported that astaxanthin forms blue salts when solutions of it are made alkaline in the absence of air. If oxygen is present, a rapid oxidation to the red astacene occurs. It seemed possible that the red carotenoid obtained from the goose-barnacle eggs might also be astaxanthin, and an attempt was made therefore to convert it to a blue salt. An alcoholic solution of the pigment was freshly prepared from the blue chromoprotein and placed in a Thunberg tube. Alcoholic KOH was then placed in the side arm and the tube evacuated. When the contents of the tube were mixed, no color change could be observed. It is possible that this negative result is due to the presence of some water in the alcoholic solution. Unfortunately, an accident invalidated an attempt

to repeat the experiment with pyridine, which appears to be the solvent Kuhn and Sørensen employed, though this is not clearly stated in their article.

The limited quantity of material available prevented further tests along this line from being made. The question of the identity of the prosthetic group of the blue chromoprotein with astaxanthin must, therefore, be left in abeyance.

During the summer of 1943, a few specimens of the goose-barnacle, *Lepas fascicularis*, were obtained. The blue pigment of the eggs of this species was found to possess properties similar to those described above. The blue chromoprotein was purified by the procedure outlined and its absorption spectrum determined with the aid of a Beckman quartz spectrophotometer.

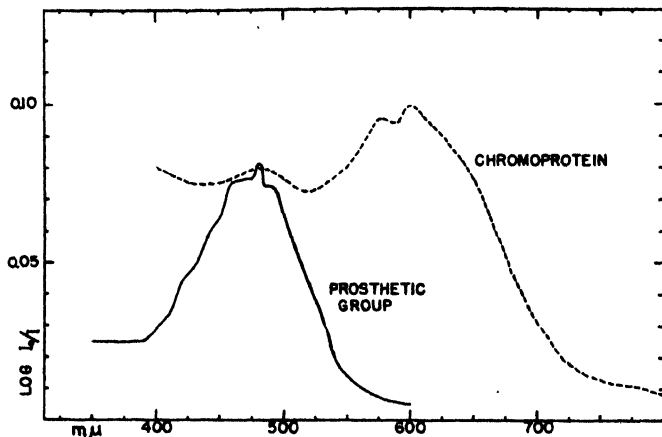


FIG. 1. Absorption curves for chromoprotein dissolved in neutral salt solution and the prosthetic group dissolved in 90 per cent ethanol.

The results are given in Fig. 1. At the end of the run, the blue pigment was precipitated with ammonium sulfate and the prosthetic group split off from the precipitate by the addition of 95 per cent ethyl alcohol. The absorption spectrum of the pink alcoholic solution was then also determined, with the results shown in Fig. 1. Owing to the limited amount of pigment available, the density values are very low and hence not too accurate. The results, however, serve to indicate the pronounced difference in absorption spectra between the chromoprotein and its prosthetic group. Maximum absorption of the chromoprotein occurs in the region λ 600 mμ, while the prosthetic group has its peak absorption in the region λ 480 mμ. There is an indication that the absorption spectrum of the prosthetic group possesses two shoulders on either side of its peak, similar to that given by Kuhn and Sørensen (1) for astaxanthin. The maximum absorption of

gested by the reversible change in color that it undergoes on the application of heat or upon acidification. Moreover, the fact that the color change upon acidification is centered at about pH 3.6 suggests that an ionizable group participating in this linkage has a pK value in this region. In this connection, it is interesting to note that the hydroxyl groups of astaxanthin possess a configuration not unlike that found in ascorbic acid, which has a pK value of 4.0.

Some linkage other than a salt type must also exist in the blue chromoprotein between the carotenoid and protein parts. This is indicated by the finding that it is possible to produce an irreversible water-soluble red product from the blue chromoprotein. This red product cannot be the free carotenoid itself since this is water-insoluble. The carotenoid, therefore, must be held in solution by a linkage to the protein other than that responsible for the blue color. The reversible color changes of the pigment that occur on heating or acidification would appear, therefore, to be due to the opening and closing of a salt-like bridge without the rupture of this second type of linkage. In the case of the formation of the irreversible water-soluble red product, some structural change must have occurred in one of the components to prevent the reformation of the salt-like bridge. Kuhn and Sørensen (1) have also postulated that a linkage other than the salt bridge type occurs between the protein and carotenoid in ovoverdin. They base this postulate on the fact that, whereas autoxidation of the blue salts of astaxanthin in pyridine occurs readily, there is no autoxidation of the carotenoid when bound to the protein.

The change in color from blue to red in the developing goose-barnacle eggs would also appear to be due to a dissociation of the protein-carotenoid complex. Since the red pigment of the eggs is not soluble in dilute salt solutions, an alteration other than a mere rupture of the salt type linkage would seem to have occurred. What changes occur in the environment of the eggs as they develop to cause this alteration or its physiological significance are unknown. The only processes that have been found *in vitro* to lead to the production of an insoluble red product are those that cause denaturation of the protein. A denaturation of the protein part of the pigment within the eggs as they develop may thus be postulated. Such a process would resemble that encountered in the mechanism of vision. According to Wald (4), the primary change from visual purple to visual yellow consists in a rupture of the carotenoid-protein complex by the action of light to liberate the carotenoid retinene. The protein component is considered to be denatured in the reaction (Mirsky (2)). What part light may be playing in the color change of the goose-barnacle eggs as they develop is not known. It would be of interest, however, to determine whether the absence of light prevents the color change in these eggs as they develop.

SUMMARY

1. The eggs of the goose-barnacle contain a blue pigment which appears to be a carotenoid-protein compound.

2. The pigment is insoluble in water, soluble in dilute salt solutions, and precipitated from solutions 42 per cent saturated with ammonium sulfate.

3. The pigment can be made to undergo a reversible color change from blue to red by the application of mild heat or acidification in the presence of ammonium sulfate. In the case of acidification, the mid-point of the color change occurs at pH 3.6.

4. Processes which cause denaturation of the protein cause an irreversible color change from blue to red with precipitation of the pigment. The formation of an irreversible but soluble red product is also described.

5. The colored prosthetic group may be extracted with alcohol and displays some of the properties of xanthophylls. Its possible identity with astaxanthin is discussed.

6. The similarity of the pigment to the green chromoprotein, ovooverdin, found in lobster eggs, is pointed out and the possible types of linkage between the protein and carotenoid portions discussed.

7. The change in color from blue to red observed in the developing eggs is attributed to a dissociation of the protein-carotenoid complex.

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FLUOROPHOTOMETRIC ANALYSIS OF VITAMIN A ESTERS*

By HARRY SOBOTKA, SUSAN KANN, AND WILHELMINE WINTERNITZ

(From the Chemistry Department, the Laboratories of the Mount Sinai Hospital, New York)

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Alcoholic solutions of esters of vitamin A display a sharp rise in the intensity of their greenish fluorescence upon ultraviolet irradiation; this behavior is in contrast to that of free vitamin A alcohol (1). This difference furnishes the basis for a method of differential quantitative analysis of vitamin A alcohol-ester mixtures by means of a fluorophotometer.

The following esters of vitamin A were studied (Fig. 1): acetate, laurate, myristate, palmitate, oleate, linoleate, and azobenzene carboxylate. Except for the last two esters, the course of the intensity of fluorescence during irradiation was found to be practically identical for equivalent concentrations. We have prepared mixtures of vitamin A alcohol with its esters in various ratios and found that the maximum of fluorescence achieved during irradiation under given conditions is an additive function of the fluorescence of vitamin A alcohol, which falls off but slightly during the first 10 minutes, and the maximal fluorescence of the ester. Hence, we have constructed a graph (Fig. 2) which gives on the ordinate the fluorescence in terms of galvanometer deflections of all mixtures of vitamin A alcohol plus acetate, the percentage of which is given on the abscissa. Individual curves are drawn for total vitamin A concentrations in multiples of 1 I.U. or 0.3 γ in the range of 0.3 to 3.3 γ per ml. In order to find the ester percentage, one traces the level of maximal galvanometer deflection from the vertical axis to the curve derived for the total vitamin A concentration used. A perpendicular line from the point of intersection leads to a point on the horizontal axis where the ester percentage can be read.

In order to apply this principle to the analysis of mixtures of vitamin A with its esters, one takes a solution of known total vitamin A concentration. In the analysis of pure substances and in the study of enzymatic hydrolysis of vitamin A esters, the concentration is known from the weight of the sample dissolved. In all other instances the total concentration may be ascertained by the Carr-Price reaction with SbCl_3 , which does not distinguish between free and combined vitamin A, but gives a measure of the total concentration. The solution is then irradiated in the fluorophotom-

* This investigation was supported by a grant from the Nutrition Foundation, Inc.

This paper is the third in a series of investigations of the fluorescence of vitamin A; for the preceding papers see (1) and (2).

eter, as described previously (1), and the course of fluorescence is followed and plotted on graph paper from minute to minute; the peak of the curve is easily recognized and the figure obtained together with the total concentration furnishes the ester percentage by the nomographic procedure outlined above.

Because of differences in the individual apparatus, such as light intensity, diaphragm opening, and density of filters, it becomes necessary to calibrate the fluorophotometer with a standard fluorescence solution of 1 part of quinine sulfate in 1,000,000 parts of acidulated water, similar to the standard used in the fluorophotometry of thiamine (3). Using the same filters as for the vitamin solutions themselves, one adjusts the galvanometer

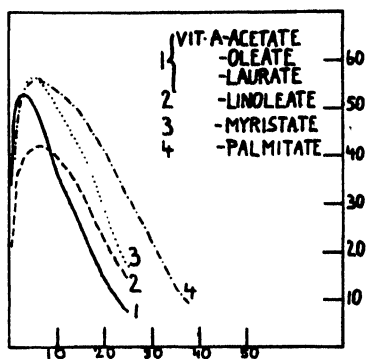


FIG. 1

FIG. 1. Fluorescence of solutions containing 3.33 γ of vitamin A in the form of various esters per ml. of ethanol. Abscissa = time of irradiation in minutes; ordinate = galvanometer deflection.

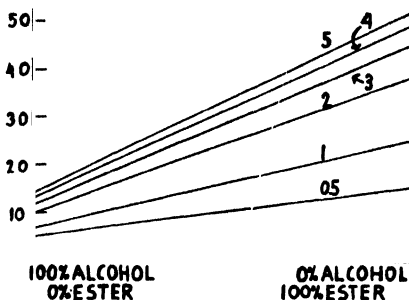


FIG. 2

FIG. 2. Nomogram for the determination of ester percentage on the abscissa for maximal galvanometer deflection G (ordinate) and total vitamin A concentrations of 0.3 γ (Curve 0.5), 0.7 γ (Curve 1), 1.3 γ (Curve 2), 2 γ (Curve 3), 2.7 γ (Curve 4), and 3.3 γ (Curve 5) in 1 ml. of absolute ethanol.

deflection produced by the fluorescence of the quinine solution to a fixed number by setting the variable resistance of the photocell-galvanometer circuit. In the present case the number 60 on the galvanometer scale was selected for the quinine standard. The calibration of fluorophotometers may also be accomplished by glass standards (4); here again, a suitable galvanometer setting must be selected for a given type of apparatus and range of intensity.

Vitamin A linoleate and vitamin A azobenzene carboxylate differ from the other esters investigated. The linoleate shows as steep an initial rise in intensity of fluorescence as do the esters of the saturated acids but the maximum is about one-fifth lower (Fig. 1, Curve 2). This difference must

be due to the acceleration of the secondary photooxidative process which destroys the highly fluorescent product of the primary photoreaction. If this photooxidation is enhanced by the catalytic influence of linoleic acid, the effect should be smaller when oxygen is driven out by a current of CO_2 . This, indeed, is the case (Table I). Whereas the maximum fluorescence intensity of the acetate is increased by 45 per cent in CO_2 (cf. (1)), that of the linoleate is increased by more than 70 per cent and the difference $G_{\text{ao.}} - G_{\text{lin.}}$ shrinks from 22 to 8 per cent.

The fluorescence curve of vitamin A azobenzene carboxylate runs an entirely different course. The galvanometer deflection rises very slowly from the initial blank value of about 8 in the course of 1 hour to 18, and after maintaining a plateau at this reading, it drops at an equally slow rate. This behavior is caused by the strong absorption of the effective radiation in the yellow solution of this ester. A similar course could be observed with any of the other esters, if one reduces substantially the intensity of irradiation.

TABLE I

Maximal Galvanometer Deflection of 3.3 γ per Ml. in Ethanol, Corrected for Fluorescence of Blank

	Without CO_2	With CO_2	Per cent increase
Vitamin A acetate	45	65	45
" " linoleate	35	60	71
Per cent difference	22	8	

EXPERIMENTAL

The method has been applied to samples of vitamin A concentrates and to fish liver oils. After the determination of the blue value by the method of Dann and Evelyn (5), an aliquot of the chloroform solution is evaporated in a current of nitrogen at 40–50° and the residue dissolved in the appropriate amount of absolute ethyl alcohol. About 12 ml. of a solution containing 0.3 to 3.3 γ per ml. of total vitamin A are required for the absorption cell (13 $\times$ 42 $\times$ 46 mm.) which is placed in the fluorophotometer.¹ The source of light consists of an 85 watt mercury capillary arc at a distance of 200 mm. from the face of the cell. The beam of light, regulated by an iris diaphragm, passes through Corning Filter 5840 (new) and through a vertical slit. At a perpendicular angle adjacent to the broad side of the cell a barrier-layer photoelectric cell responds to the fluorescent light emanating from the solution and filtered through Corning Filter 3389 (new) which excludes scattered ultraviolet light. The photoelectric current is

¹ We have used the fluorophotometer of Pfaltz and Bauer, Inc., New York.

measured by a multiple mirror galvanometer with variable resistance and with a sensitivity of 2.3×10^{-9} ampere per mm. Table II is a summary of results obtained by this procedure.

Correction for Linoleic Acid—If it is known that L per cent of vitamin A in a sample is present in the form of the linoleate, the galvanometer reading G may be extrapolated to the value $G' = G/(1 - 0.0022L)$, the galvanometer deflection that would be read if the linoleic acid was replaced by a saturated fatty acid. If the linoleic acid content is unknown, one may assume that the share of linoleic acid and other unsaturated acids with two or more

TABLE II
Ester Percentage of Vitamin A Preparations

Preparation	Total vitamin A		Per cent of esterified vitamin A	
			Found	Corrected for 20 per cent linoleate
	<i>I. U. per gm.</i>	<i>γ per gm.</i>		
Vitamin A alcohol, crystalline	3,200,000	1,000,000	0	
“ “ acetate, “	2,800,000	870,000	100	
Commercial vitamin A Concentrates 11, 16, 18	$\begin{cases} 700,000 \\ -900,000 \end{cases}$	$\begin{cases} 210,000 \\ -300,000 \end{cases}$	10-15	
Same, Concentrate 46	650,000	195,000	30	31.5
Saponified concentrate	600,000	185,000	0	
Vitamin A in sterile-sesame oil	500,000	155,000	73	76
Standard concentrate	200,000	60,000	82	86.5
Concentrate in capsules	200,000	60,000	67	70
			75	78
Oleum percomorphum	60,000	18,000	55	58
			47.5	49
Halibut liver oil	60,000	18,000	54	57
			59	62
U. S. P. cod liver oil, No. 2	1,700	520	60	63.5
Normal rat liver	330	105	80	

double bonds is the same for the vitamin A fraction as for the whole oil. An estimated average of 20 to 25 per cent (6), multiplied by the per cent of uncorrected ester, yields L and hence, according to the above formula, a correction factor >0.945 by which the observed galvanometer deflection must be divided in order to obtain G' for the final nomographic evaluation.

Biological Specimens—The method lends itself to the study of material from experimental animals, such as rat liver (Table II) which is relatively low in carotenoid content. In the application of the method to the differential analysis of vitamin A esters in blood serum, the interfering effect of carotenoids makes itself felt. Both carotene and the hypophasic caro-

tenoids reduce the transmission of ultraviolet light by the extract, and also display fluorescence of their own. Their chromatographic removal from vitamin A solutions with quantitative recovery of the latter in the filtrate or eluate is not feasible.

We are indebted to Dr. J. G. Baxter of Distillation Products, Inc., for samples of the various esters used in this investigation.

SUMMARY

The difference in the course of fluorescence between vitamin A alcohol and vitamin A esters has been utilized for the differential analysis of their mixtures. The results obtained by fluorophotometry are evaluated by a nomographic procedure. A series of esters has been studied and certain exceptions in their behavior considered. Examples are given for the determination of ester percentage in a number of vitamin A preparations.

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THE VERATRINE ALKALOIDS

XXI. THE CONVERSION OF RUBIJERVINE TO ALLORUBIJERVINE. THE STEROL RING SYSTEM OF RUBIJERVINE

By WALTER A. JACOBS AND LYMAN C. CRAIG

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

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The basis for the correlation of the veratrine bases with the alkaloids of *Solanum* and with the sterols has been presented previously (1). The failure, however, to obtain any indication of the formation of Diels' hydrocarbon on dehydrogenation from any one of the veratrine bases thus far examined in contrast to the experience with solanidine suggested that this might be due to some difference in ring structure. This point has been discussed in earlier communications and certain evidence was presented which suggested the possibility that Ring B of the veratrine alkalamines could be 5-membered, instead of 6-membered as in the sterols. However, in the light of results which we have now obtained with rubijervine, it has been shown that this base parallels the behavior of solanidine in certain reactions which can be best explained on the assumption that Ring B is 6-membered. Rubijervine, therefore, like solanidine, must possess the regular steroidal skeleton.

The method of Sexton (2) for the conversion of cholesterol to cholestenone, which was employed by Schöpf and Hermann (3) for the preparation of solanidone from solanidine, has now been applied to rubijervine. The ketone *rubijervone* was readily obtained and was characterized by its *oxime*. Rubijervone was then subjected to the action of aluminum isopropylate, essentially as used by Schoenheimer and Evans (4) for the conversion of cholestenone to allocholesterol and its epimer. From the reaction mixture, a substance was obtained which was isomeric with rubijervine. As in the case of the cholestenone reaction, it is probable that a mixture of two substances was produced; *viz.*, *allorubijervine* and *epiallorubijervine*. The substance obtained by recrystallization yielded with digitonin, after several days standing, a relatively small amount of precipitate which, on still longer standing, was followed by a gradual deposition of larger crystals. At any rate, the precipitability by digitonin appeared much less marked than in the case of rubijervine itself. Unfortunately, the amount of material available was too small to make possible an attempt to fractionate the reaction product into pure *allorubijervine* and *epiallorubijervine* over the digitonide.

But of greatest significance was the fact that this material now gave a pronounced Rosenheim reaction in contrast to rubijervine itself, thus

paralleling the difference in behavior in this respect of allocholesterol and cholesterol. The conclusion must, therefore, be drawn that the transformation of rubijervine to allorubijervine is to be explained on the same basis as that of cholesterol to allocholesterol, in which the double bond of the former shifts from the 5,6 position to the 4,5 position, and is due to conjugation with the carbonyl group in the intermediate rubijervone. Such an interpretation, barring an improbable rearrangement, would require that carbon atom 5 in rubijervine cannot carry an angular methyl group, as we have originally suggested for the other veratrine alkamines, and Ring B must, therefore, be 6-membered.

It is apparent that a different reason must be sought for the failure of rubijervine to yield Diels' hydrocarbon on dehydrogenation than the possibility that carbon atom 5 bears an angular methyl group and that Ring B is 5-membered.

The attempt was made to subject cevine to an analogous series of reactions but, unfortunately, no crystalline carbonyl derivative could be obtained after the alkamine was heated with copper powder. For the time being at least, a final decision cannot be made as to whether Ring B in this alkamine, and therefore in the remaining veratrine bases, possesses the regular sterol structure or a variation of it. But if the regular arrangement should be found to be the case, the origin of the hexanetetracarboxylic acid obtained from cevine and germine, as well as that of the series of fluorene hydrocarbons obtained on dehydrogenation, will require reinterpretation.

EXPERIMENTAL

Rubijervone—An intimate mixture of 1 gm. of rubijervine with 4 gm. of copper powder was placed in a sublimation apparatus, and air was removed with CO₂. The mixture was gradually heated at atmospheric pressure from 150° to 200° in the course of 15 minutes, during which water of crystallization was driven off. During an additional 15 minutes, the temperature was raised from 200° to 290°, and the mixture was allowed to cool. The apparatus was then exhausted to about 0.1 mm., and the mixture was again heated finally to 290° during a period of about 1 hour. An appreciable sublimate collected on the condenser and was colored by copper. The yield was 0.75 gm. When this was dissolved in 95 per cent alcohol, a red color, apparently due to copper, resulted, which was partly removed by treating the solution with norit. The filtrate, on concentration to about 5 cc., yielded a copious crop of almost colorless needles (0.38 gm.) which gradually melted at 198–201°, but did not clear till 202° (uncorrected). On further recrystallization, it melted at 202–204° after slight preliminary sintering.

C₂₇H₄₁O₂N. Calculated, C 78.77, H 10.05; found, C 78.59, H 10.30

The *oxime* was prepared under conditions essentially as described by Schöpf and Hermann (3) for solanidone oxime, with the exception that a very small volume of methanol was added to produce an initial clear solution. The reaction mixture gradually formed a thick paste of crystals of the hydrochloride of the oxime. After standing at room temperature overnight, the collected salt was decomposed in aqueous suspension with dilute sodium carbonate and the base was extracted with chloroform. After concentration, the oxime base was obtained from alcohol as lustrous flat needles and was then recrystallized several times from methanol. Most of it appeared to melt at 160°, but crystallized again and then melted at about 247–254°, depending on the rate of heating.

$C_{27}H_{42}O_2N_2$. Calculated. C 75.99, H 9.93, N 6.57
Found. " 75.85, " 9.77, " 6.59

Allorubijervine (*Epiallorubijervine*)—Because of the small amount of material used for this reaction, somewhat different conditions were employed from those given by Schoenheimer and Evans (4) for the reduction of $\cdot$ choستنone. Aluminum isopropylate was prepared according to Adkins (5) by refluxing 1 gm. of aluminum leaves, after amalgamation, with 15 cc. of absolute isopropanol until dissolved. To this was added 0.2 gm. of rubijervone, and the protected mixture was refluxed for 22 hours. Towards the end, a small portion of the solvent was allowed to boil off. The chilled reaction mixture was decomposed with excess 20 per cent potassium hydroxide and extracted with ether. The dried extract after concentration was boiled down with 95 per cent alcohol to remove isopropanol. The concentrated solution crystallized and, after chilling, was collected at 0°. After recrystallization from 95 per cent alcohol, it formed needles which gradually softened to a melt at 218–222°. The substance gave a purple color with trichloroacetic acid.

$C_{27}H_{42}O_2N$. Calculated, C 78.38, H 10.49; found, C 78.40, H 10.70

The question of the homogeneity of this substance cannot be decided until more material is available, and it is probable that, for separation into *allorubijervine* and *epiallorubijervine*, conversion into the digitonide will be necessary.

All analyses were performed by Mr. D. Rigakos of this laboratory.

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THE ACONITE ALKALOIDS

XIII. THE ISOLATION OF PIMANTHRENE FROM THE DEHYDROGENATION PRODUCTS OF STAPHISINE

BY LYMAN C. CRAIG AND WALTER A. JACOBS

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

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In the course of our work on the simpler, less oxygenated alkaloids of the aconite series, the possibility of a relationship to another class of natural products, the terpenes and perhaps in particular the diterpenes, has steadily become more apparent. A certain resemblance had been evident for some time in the general nature of the hydrocarbons which were encountered in the dehydrogenation of atisine (1), napelline (2), and of staphisine (3), since these appeared to be mainly phenanthrene hydrocarbons, and no hydrocarbons with larger ring systems were isolated. None of these hydrocarbons, with the exception of 1-methylphenanthrene from atisine, was definitely identified. Further progress along this line has been greatly hindered by the lack in this laboratory of the proper phenanthrene hydrocarbons with which to make the necessary direct comparisons.

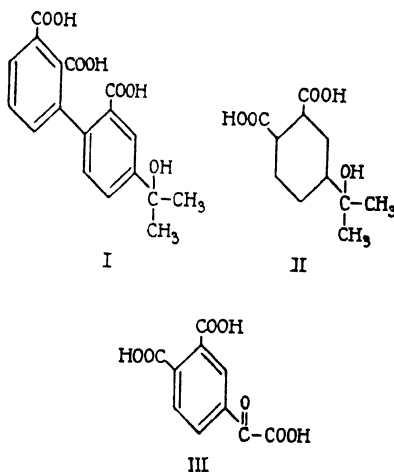
In the interest of further examination of the suggested resemblance to the diterpenes, we have carried out a dehydrogenation of commercial abietic acid, possibly contaminated with *d*-pimaric acid, under the same conditions as were used for our alkaloids. Fractionation of the resulting hydrocarbons gave a slightly different picture, since considerably larger amounts of more volatile hydrocarbons were obtained. However, aside from retene, which was the principal hydrocarbon, a small amount of a hydrocarbon with the empirical formula $C_{16}H_{14}$ was isolated. The physical properties of this hydrocarbon, as well as of its picrate and styphnate, were in good agreement with the recorded properties of 1,7-dimethylphenanthrene or pimanthrene, and there can be no doubt of its identity because of its source.

A corresponding $C_{16}H_{14}$ phenanthrene derivative was reported previously (3) as a product of the dehydrogenation of staphisine, although the melting point was a few degrees lower than that recorded for pimanthrene. However, we have now further purified this hydrocarbon and found it to agree in all respects with the hydrocarbon from abietic acid. No depression in the mixed melting point was found. The picrates and styphnates from both sources were likewise indistinguishable. Their identity would appear to afford the first direct evidence in support of the possibility of a relationship between the diterpenes and the aconite alkalamines.

In order to gain further information in regard to the nature of the hydro-

carbons obtained in the dehydrogenation of staphisine, a type of oxidative procedure has been attempted which proved so useful in the establishment of the structures of retene and pimanthrene, and thus of the diterpenes, abietic and *d*-pimaric acids, respectively (4). Exact similarity in behavior has not been found, but nevertheless certain resemblances have been encountered. Oxidation of the main hydrocarbon from staphisine, the hydrocarbon $C_{19}H_{20}$, first to the quinone with chromic acid and subsequently with potassium permanganate, did not lead to the isolation of the hydroxyisopropylidiphenyltricarboxylic acid, $C_{19}H_{16}O_7$ (Formula I), which is formed from retene by such a procedure. Instead, a simpler acid, $C_{11}H_{12}O_6$, apparently a hydroxyisopropylphthalic acid, was isolated.

The formation of this substance would indicate that the $C_{19}H_{20}$ hydrocarbon bears on one of its rings an isopropyl group, such as occurs in retene,



and no other substituent on the same ring of the phenanthrene nucleus. A probable position of this isopropyl group, which could lead to the hydroxyisopropyl group of the structure represented in Formula II, may be inferred from the fact that pimanthrene is also formed during the same dehydrogenation.

If the above conclusions are assumed to be correct, 2 carbon atoms would remain to be accounted for in the $C_{19}H_{20}$ hydrocarbon. It may be inferred that one of these is located on position 1, if it is involved in the formation of 1,7-dimethylphenanthrene from staphisine. The remaining carbon cannot be joined to this carbon atom to form an ethyl group on position 1, since such a substance, homoretene, is known (5). The latter gives a quinone which melts much lower (over 60°) than the quinone from our $C_{19}H_{20}$ hydrocarbon. Therefore, it appears probable that the $C_{19}H_{20}$

hydrocarbon is a methylretene in which the second methyl group is attached to one of the positions, 2, 3, or 4.

With the hope that the hydroxyisopropylphenyldicarboxylic acid postulated above could be found also among the oxidation products of retene, and therefore aid in establishing its exact identity, we have repeated the oxidation of retenequinone essentially as described by Bucher (6), and by Ruzicka, de Graaff, and Hosking (4), on a small sample of the quinone which was available. This gave at once without difficulty the hydroxyisopropyl-diphenyltricarboxylic acid reported by them as a major product, but the above hydroxyisopropylphenyldicarboxylic acid was not found in the mother liquors. However, another acid was isolated as the trimethyl ester, which we wish to record at this point, since it was apparently also encountered in our study of atisine to be described in a subsequent paper. After esterification of the final crude mother liquors with diazomethane, fractionation yielded a crystalline substance, $C_{13}H_{12}O_7$, which from the analytical data appeared to be the trimethyl ester of a dicarboxyphenylglyoxylic acid, $C_{10}H_8O_7$. Although we have not had opportunity as yet to establish the exact identity of this acid, it might be expected to result from the further oxidation of the above hydroxyisopropyl-diphenyltricarboxylic acid. Such α -keto acids have been repeatedly encountered in the oxidation of aromatic hydrocarbons. This acid could possess the structure shown in Formula III. This would be consistent with the simultaneous production of both trimellitic and hemimellitic acids, which have been found to be formed (4) during the same oxidation.

EXPERIMENTAL

Dehydrogenation of Abietic Acid—25 gm. of commercial abietic acid were well mixed with 75 gm. of selenium, and the mixture was heated in an apparatus such as that previously employed and through which a steady stream of nitrogen was passed. The temperature was gradually raised to 340° during 30 minutes, and held at this point for 2 hours.

The volatile material was discarded. The finely pulverized residue was well extracted with ether. After evaporation of the solvent, a residue of 15 gm. was obtained. This residue was dissolved in benzene and chromatographed through 200 gm. of activated alumina. 11.7 gm. of a hydrocarbon fraction passed through the column in the first and main band.

This material from the first band was then fractionated in a sublimation apparatus under reduced pressure and separated into four fractions, which consisted of 2.6, 3.9, 3.3, and 0.7 gm., respectively. The last three fractions were mostly crystalline. The first fraction (2.6 gm.) with 1 gm. of the second fraction was placed in a micro fractionating column 20 cm. in length and separated into twenty-five fractions of roughly 130 mg. each. The

first eighteen fractions were non-crystallizing oils of high hydrogen content, from which Fractions 17 and 18 yielded pimanthrene. The later fractions which crystallized were mostly retene. This was readily obtained by recrystallization.

Isolation of Pimanthrene—Fraction 17 (approximately 130 mg.) was treated with 100 mg. of picric acid in benzene. The picrate was then recrystallized from acetone. 60 mg. of fine yellow needles were obtained, which melted at 131–133°.

$C_{18}H_{14} \cdot C_6H_3O_7N_3$. Calculated, C 60.66, H 3.93; found, C 60.88, H 4.06

Fraction 18 behaved in a similar way, but neither Fraction 15 nor 19 yielded a homogeneous picrate by such treatment. Only material with a very unsatisfactory melting point was obtained.

The pimanthrene regenerated from the pure picrate after recrystallization from ether formed heavy characteristic plates which melted at 84–85°.

$C_{18}H_{14}$. Calculated, C 93.15, H 6.85; found, C 93.30, H 7.24

A portion of the hydrocarbon, when treated with an equivalent of styphnic acid in acetone, yielded characteristic needles of the styphnate. After recrystallization from acetone-ether, it melted at 162–164°.

$C_{18}H_{14} \cdot C_6H_3O_8N_3$. Calculated, C 58.54, H 3.80; found, C 58.32, H 3.95

Dimethylphenanthrene from Staphisine—The small amount of so called dimethylphenanthrene previously recorded (3), which was obtained from Fraction 4 of the fractionated hydrocarbons from staphisine, and which was reported to melt at 78–81°, has since been recrystallized from ether. It has now been found to melt at 84–85°, and formed the same characteristic large plates observed in the case of pimanthrene obtained above from abietic acid. A mixed melting point showed no depression. The picrates prepared from the hydrocarbons from both sources also agreed in properties, and a mixed melting point (131–133°) showed no depression.

For further comparison, the styphnate was prepared from the staphisine hydrocarbon. The same characteristic crystals formed from acetone, exhibited by the styphnate from pimanthrene and after recrystallization from acetone-ether, melted at 162–164°. A mixed melting point with pimanthrene styphnate showed no depression.

$C_{18}H_{14} \cdot C_6H_3O_8N_3$. Calculated, C 58.54, H 3.80; found, C 58.81, H 4.08

Oxidation of $C_{19}H_{20}$ Hydrocarbon from Staphisine. The Quinone, $C_{19}H_{18}O_2$ —The crystalline material obtained on recrystallization of Fractions 15 and 16 of the hydrocarbons from staphisine previously described (3) were recombined with their mother liquors, and in this way a total of 295 mg. of crude hydrocarbon was obtained. It was dissolved in 3.9 cc. of glacial

acetic acid and treated with 0.6 gm. of chromic oxide dissolved in 0.6 cc. of water. The mixture was heated on the steam bath for 6 hours, and then allowed to crystallize at 0° overnight. 90 mg. of stout, orange-colored needles were collected with cold acetic acid. After recrystallization from acetone, the quinone melted at 213–216°.

$C_{18}H_{18}O_2$. Calculated, C 81.97, H 6.52; found, C 81.72, H 6.68

Oxidation of Quinone, $C_{18}H_{18}O_2$. Hydroxyisopropylphthalic Acid (?)—Approximately 80 mg. of the above quinone were oxidized according to the directions of Bucher (6). Ether extraction of the acidic oxidation products gave a total of 90 mg. of partially crystalline material. On recrystallization from ether, 47 mg. slowly separated. The substance melted with effervescence at about 170°, depending somewhat on the rate of heating. At a higher temperature, the melt appeared to crystallize again almost entirely, presumably as the anhydride, and then melted at about 290–294°. This behavior did not change upon recrystallization.

$C_{11}H_{12}O_6$. Calculated, C 58.90, H 5.40; found, C 59.08, H 5.62

1.040 mg. of the acid, when titrated against phenolphthalein with 0.1035 N NaOH, required 0.0870 cc. Calculated for 2 equivalents, 0.0896 cc.

Oxidation of Retenequinone with Potassium Permanganate. The Ester, $C_{13}H_{12}O_7$ —600 mg. of retenequinone were oxidized under the same conditions as were used for the previous quinone. 540 mg. of acidic material were extracted with ether. This crystallized mainly from acetone-ether to give the hydroxyisopropylidiphenyltricarboxylic acid described previously (4), which melted with some effervescence at 186–192°, depending somewhat on the rate of heating.

$C_{18}H_{18}O_7$. Calculated, C 62.78, H 4.64; found, C 62.87, H 4.80

After collection of all the readily crystallizable material, the mother liquor was esterified in acetone solution with diazomethane. The esters would not crystallize directly and were separated into three fractions by fractional distillation under 0.2 mm. pressure. The fractions weighed 80, 70, and 30 mg., respectively, and distilled from an oil bath held at temperatures varying from 180–220°.

The first fraction crystallized readily from ether and upon recrystallization from acetone-ether yielded 14 mg. of well formed, heavy plates which melted at 149–151°.

$C_{13}H_{12}O_7$. Calculated. C 55.70, H 4.32, OCH_3 33.20
Found. " 55.82, " 4.47, " 33.2

These figures suggest the trimethyl ester of a dicarboxyphenylglyoxylic acid.

All of the analytical data were obtained by Mr. D. Rigakos of this laboratory.

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THE ACONITE ALKALOIDS

XIV. OXIDATION OF THE HYDROCARBON FROM THE DEHYDROGENATION OF ATISINE

By LYMAN C. CRAIG AND WALTER A. JACOBS

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

(Received for publication, December 14, 1943)

In structural studies on the aconite alkaloid, atisine, Lawson and Topps (1) isolated a hydrocarbon, $C_{17}H_{16}$, which they presumed to be an alkyl-phenanthrene derivative, by selenium dehydrogenation of the alkaloid. This observation was later confirmed by us (2), and the hydrocarbon was definitely shown to be a phenanthrene hydrocarbon by absorption spectra studies. This hydrocarbon, which is the main product formed during the dehydrogenation, does not appear thus far to have been encountered as a degradation product from any other naturally occurring material so far as we have been able to learn from the literature. * Degradation experiments have, therefore, been necessary to determine the number and positions of the substituting alkyl groups. For this purpose, the type of oxidative procedures which proved so useful in the elucidation of the structures of retene and of pimanthrene in the diterpene series appeared to offer the most promise. The results of such experiments, performed with very limited material, are reported here.

The hydrocarbon was found upon oxidation with chromic acid in glacial acetic acid solution to give in good yield an orange-colored quinone, $C_{17}H_{14}O_2$, which melted at 149–151°. This behavior indicated that positions 9 and 10 of the phenanthrene nucleus were unsubstituted.

The crystalline quinone was further oxidized with potassium permanganate according to the directions given by Bucher (3) for the oxidation of retenequinone. This resulted in a mixture of acids which consisted mainly of a tetracarboxylic acid, evidently a *diphenyltetracarboxylic acid* from the analytical data. This acid was formed in good yield from the quinone, and crystallized readily from acetone and ether. It appeared to melt with decomposition and sublimation at about 340–345°. It is possible that anhydride formation occurred during the melting point determination, since the melting point of a substance described below, which analytical data indicated to be the anhydride, was the same as that of the acid. This acid was found to condense readily with resorcinol to give a characteristic fluorescein dye, and thus the presence of two adjacent carboxyl groups was indicated. The formation of such a tetracarboxylic acid shows that the original phenanthrene hydrocarbon contained two alkyl groups.

The acid formed a crystalline *tetramethyl ester* which melted at 149–150°. Hydrolytic experiments with the ester indicated the presence of three ester groups which were readily saponified with alkali, and one which was much more resistant. A crystalline *monomethyl ester*, therefore, could be isolated from the saponification mixture which melted at 338–341°.

An attempt was made to oxidize the acid further with fuming nitric acid and a little manganous nitrate by the method used by Bucher (3) to accomplish the oxidation of the diphenyl acids to the benzenepolycarboxylic acids. The main product of the oxidation, which was isolated in a yield of over 56 per cent of the theoretical, proved to be a substance which gave analytical data for a *monoanhydride* of the original acid. It melted at the same point as the acid itself.

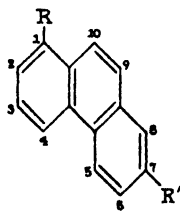
The material contained in the mother liquors from the above tetracarboxylic acid was esterified with diazomethane. When the resulting mixture was fractionally distilled, a crystalline ester could be isolated from one of the fractions. This proved to be different from the ester of the above tetracarboxylic acid, which was also isolated from the mixture. Sufficient of the former crystalline ester was not available for final purification, but the substance melted at 93–98°, and gave good analytical figures for the ester of a benzenetricarboxylic acid. The ester of 1,2,3-benzenetricarboxylic acid has been reported to melt at 100–101° (4), that of the 1,3,5 isomer at 144° (5), while the ester of the remaining possibility, the 1,2,4 isomer, has been reported as an oil. Our data with the substance in question, therefore, strongly point to the ester of hemimellitic acid.

Upon evaporation of the mother liquors from this acid, a thick oil was obtained. On hydrolysis with strong HCl, this yielded a small amount of a crystalline acid. Here again, there was insufficient material for final purification, but the analysis clearly indicated a benzenetricarboxylic acid. The melting point was approximately 220–227°. The melting points of the possible isomers, hemimellitic, trimellitic, and trimesic acids, are 190°, 228°, and 380°, respectively. The substance could not, therefore, have been hemimellitic acid. Because of the unlikelihood that trimesic acid could be a product of the oxidation of a phenanthrene and because of the melting point discrepancy as well, this acid would appear to be excluded. It was most probably trimellitic acid.

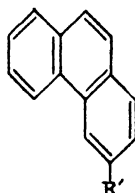
The acid aqueous layer which remained after the ether extraction of the oxidation products was also examined, and from this an appreciable amount of material was obtained as a mixture of esters. From the latter, some of the tetramethyl ester of the tetracarboxylic acid reported above was isolated, and in addition a small amount of a crystalline ester was obtained, which melted at 148–149°. The analytical data and the melting point of the latter agreed well with the data obtained with the ester of the acid

previously found in the oxidation of retenequinone (6), and which has been interpreted as a dicarboxyphenylglyoxylic acid. A mixed melting point of the esters obtained from both sources showed no depression.

If the above conclusion, that hemimellitic acid and trimellitic acid result from the oxidation of the quinone, is correct, two possibilities are suggested in regard to the identity of the phenanthrene hydrocarbon, as shown in Formulas I and II. In such a case, similarity would be offered to the behavior of the quinone derived from *d*-pimaric acid (4) when it is subjected to this type of oxidation. This raises the question whether the above tetracarboxylic acid from atisine is not identical with the 2,3,2',4'-diphenyltetracarboxylic acid, which is the main product formed by the oxidation of pimanthrenequinone. The melting point of the ester of the acid derived from atisine was found to be 149–150°, and that reported for the tetramethyl ester of diphenyl-2,3,2',4'-tetracarboxylic acid is 153–154° (4). However, while the free acid obtained by us crystallized readily and



Formula I



Formula II

melted at 340–345°, Ruzicka, de Graaff, and Hosking did not report the crystalline character of their acid from pimanthrenequinone.

If our acid should prove to be diphenyl-2,3,2',4'-tetracarboxylic acid, it would indicate that atisine has much of the structure of the abietic or pimaric acid group of diterpenes, and would support the conclusion that the hydrocarbon melting at 41–43° is a pimanthrene homologue; *viz.*, either 1-ethyl-7-methylphenanthrene or 1-methyl-7-ethylphenanthrene. However, these two substances have been reported to melt at 81° (7) and 87.5° (8), respectively. A comparison of melting points of these hydrocarbons with the data obtained with the $C_{17}H_{16}$ hydrocarbon from atisine is given in Table I. The divergence in properties appears to be sufficient to preclude the identity of the atisine hydrocarbon with either of these methylethylphenanthrenes, and the conclusion is, therefore, necessary that another isomer must be in question. This would indicate that the diphenyltetracarboxylic acid from atisine could not be identical with the one obtained on oxidation of pimanthrenequinone.

Among the products of the dehydrogenation of atisine reported by us (2) is 1-methylphenanthrene. It could, therefore, be inferred that the same position is occupied by one of the alkyl groups in the methylethylphenanthrene obtained from this alkaloid. This must necessarily be the case if this hydrocarbon could furnish 1,2,3-benzenetricarboxylic acid upon oxidation. There appear to be only two positions now possible for the remaining substituent in this hydrocarbon if it is to yield on oxidative degradation the 1,2,4-benzenetricarboxylic acid discussed above. These are positions 6 and 7. Position 7 appears excluded on the basis of the above comparison with the 1,7-methylethylphenanthrene and its isomer. There remains, therefore, the possibility that the hydrocarbon is either 1-methyl-6-ethylphenanthrene or 1-ethyl-6-methylphenanthrene. Neither of these compounds has been reported in the literature so far as we are aware, and final confirmation of identity by direct comparison must await the opportunity to obtain these substances synthetically.

TABLE I
Comparative Melting Point Data of $C_{17}H_{16}$ Hydrocarbon

	Hydrocarbon	Picrate	Trinitrobenzene derivative	Quinone
	°C.	°C.	°C.	°C.
1-Methyl-7-ethylphenanthrene	87.5		134	
1-Ethyl-7-methylphenanthrene	81	115-116		154-155
$C_{17}H_{16}$ hydrocarbon from atisine	41-43	129-131	145-148	149-151

In the preceding paper of this series (6), evidence was presented to show that a 1,7-disubstituted phenanthrene, pimanthrene, is present among the dehydrogenation products of staphisine, the alkaloid from *Delphinium staphisagria*, which demonstrated its probable diterpenoid character. In similar manner, the above experiments with atisine may be taken as strongly suggestive of its diterpenoid nature. However, from the above discussion, an interesting variation in the usual diterpenoid structure would be offered if it should be confirmed that the 6 position of the perhydrophenanthrene ring system is involved instead of position 7, as in the case of the regular diterpenes. If position 6 originally bears an isopropyl group, it would still be compatible with the isoprene rule.

On an empirical basis, there has been the suggestion that atisine (2) is of pentacyclic character, since the presence of two double bonds can be shown by hydrogenation. The perhydrophenanthrene ring system would account for three of the rings, while a fourth ring must contain the nitrogen atom. It is still possible that the "fifth ring" may be accounted for by an isolated

double bond which has resisted hydrogenation procedures and has thereby escaped detection. If this should prove to be so, atisine would be of tetracyclic character. This is a question under further study.

Because of the laborious procedure required to obtain the $C_{17}H_{16}$ hydrocarbon from atisine, itself an alkaloid available only with difficulty, and because of the intricacies of the fractionation processes involved, some of the results and conclusions presented can be regarded only as tentative and will require confirmation whenever possible. They are, however, now presented because of their importance to the difficult problem of the structure of this group of alkaloids.

EXPERIMENTAL

The Quinone, $C_{17}H_{14}O_2$, from the $C_{17}H_{16}$ Hydrocarbon—A solution of 137 mg. of the hydrocarbon in 0.40 cc. of acetic acid was treated with 0.27 gm. of chromic oxide dissolved in 0.27 cc. of water and 1.37 cc. of acetic acid. The mixture was heated on the steam bath for 7 hours and then left at 0° overnight. 50 mg. of orange-yellow crystals were collected with a little cold acetone. After recrystallization from acetone, the substance melted at 149 – 151° .

$C_{17}H_{14}O_2$. Calculated, C 81.57, H 5.63; found, C 81.40, H 5.88

Oxidation of Quinone with Potassium Permanganate. Diphenyltetracarboxylic Acid—390 mg. of the quinone were oxidized with permanganate according to the directions of Bucher (3). After removal of excess permanganate, the concentrated and acidified solution was exhaustively extracted with ether. The acid layer was set aside to be treated further, as described below. The ether extracts gave a crystalline residue of 325 mg. After recrystallization from acetone, 195 mg. of well formed needles were obtained which melted at 340 – 345° , depending somewhat on the rate of heating. There was a certain amount of decomposition and sublimation at the melting point.

$C_{18}H_{10}O_6$. Calculated, C 58.17, H 3.05; found, C 58.30, H 3.21

When a mixture of the acid with an equivalent of resorcinol and a drop of H_2SO_4 was heated for a few minutes at 160° , a reddish color developed. On treatment of the product with dilute NaOH, the brilliant and typical greenish yellow fluorescence of a fluorescein was apparent.

90 mg. of the tetracarboxylic acid were esterified in acetone solution with diazomethane. 82 mg. of well formed plates or prisms of the tetramethyl ester crystallized from ether. After recrystallization from benzene, the melting point remained unchanged at 149 – 150° .

$C_{26}H_{18}O_8$. Calculated. C 62.15, H 4.66, OCH_3 32.11
 Found. " 62.11, " 5.00, " 32.40

0.1425 gm. of the tetramethyl ester was refluxed in a mixture of 2.000 cc. of 1.005 N NaOH and 2.0 cc. of methanol for 75 minutes. The solution was titrated back against phenolphthalein with HCl. 1.210 cc. of 0.1 N alkali were consumed. Calculated for 3 equivalents, 1.252 cc.

The titration mixture was concentrated to remove methanol, and acidified with HCl. The acid was extracted with ether and crystallized from acetone. It melted at 338–341°, and proved to be the monomethyl ester.

$C_{17}H_{12}O_8$. Calculated. C 59.28, H 3.52, OCH_3 9.00
 Found. " 59.45, " 3.69, " 9.11

Attempted Oxidation of Diphenyltetracarboxylic Acid. The Monoanhydride (?)—100 mg. of the acid were treated in a short test-tube with a small drop of 50 per cent manganous nitrate and 0.5 cc. of HNO_3 (1.5). The mixture was heated on the steam bath until the volume was reduced to about half and then more HNO_3 was added. After being heated for an hour longer, the mixture was allowed to crystallize at room temperature overnight. 53 mg. were collected with a few drops of HNO_3 . It crystallized from acetone in fine short needles which melted at 338–340°, and on analysis was found to be the monoanhydride.

$C_{16}H_8O_7$. Calculated, C 61.52, H 2.58; found, C 61.28, H 2.75

1.21 mg. of substance were heated with 0.200 cc. of 0.1035 N NaOH on the steam bath for 2 hours, and titrated back against phenolphthalein. 0.145 cc. was consumed. Calculated for 4 equivalents, 0.150 cc.

Benzenetricarboxylic Trimethyl Ester (Hemimellitic Ester?)—The mother liquors from the crystallization of the above diphenyltetracarboxylic acid were combined and esterified in acetone with diazomethane. The resulting mixture of esters, which amounted to approximately 420 mg., was fractionated at 0.2 mm. Three approximately equal fractions were obtained, of which the second and third appeared to consist mainly of the tetramethyl ester of the above tetracarboxylic acid. However, the first fraction yielded other material which could be obtained by fractional crystallization from acetone and ether, and which melted at 93–98°. There was insufficient material for final purification. The melting point of the hemimellitic trimethyl ester has been reported as 100–101° (4).

$C_{12}H_{12}O_6$. Calculated, C 57.12, H 4.80; found, C 57.04, H 4.88

Benzenetricarboxylic Acid (Trimellitic Acid?)—Other crystalline material was obtained from Fraction 1 with a higher melting point, which appeared to consist mainly of the ester of the tetracarboxylic acid. However, the mother liquors of the above benzenetricarboxylic acid also yielded material

which would not crystallize. This amounted to about 75 mg. or almost half the fraction. It was a rather mobile oil which distilled readily at 100° at 0.2 mm. It was heated at 110° in a sealed tube with 2 cc. of HCl (1.19) for 2 hours. The concentrated mixture yielded a slightly brown residue from which some of the color was removed with bone-black in acetone. Fractional crystallization from acetone and benzene gave a small amount of well defined, short, stout needles which sintered at 217° and melted at 220–227°. There was insufficient material for further purification. The melting point of trimellitic acid is given as 228° (4).

$C_6H_6O_6$. Calculated, C 51.42, H 2.88; found, C 51.44, H 3.03

Investigation of Acid Aqueous Layer from Permanganate Oxidation. The Ester, $C_{13}H_{12}O_7$ —The aqueous layer which remained after ether extraction of the acid oxidation products was treated with excess sodium carbonate and evaporated to dryness. Since the residue, although mostly an inorganic mixture, still contained organic material, it was suspended in acetone and treated with excess diazomethane. After filtration, the filtrate on concentration gave a residue which was dissolved in ether and again filtered. The filtrate now yielded 114 mg. of residue, which proved to be a mixture of esters. Two substances were isolated from this by fractional crystallization from acetone and from benzene. One of these proved to be the ester of the above diphenyltetracarboxylic acid. The other ester, although with almost the identical melting point, proved to be a different substance. It crystallized from acetone or benzene in hexagonal platelets which melted at 148–149°. The substance was found to be identical with a substance obtained in almost the same way by the oxidation of retene, as reported in Paper XIII of this series (6). It is apparently the trimethyl ester of a dicarboxyphenylglyoxylic acid. A mixed melting point showed no depression.

$C_{13}H_{12}O_7$. Calculated, C 55.70, H 4.32; found, C 56.02, H 4.44

All analyses were performed by Mr. D. Rigakos of this laboratory.

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NOTE ON THE TOTAL OSMOTIC ACTIVITY OF HUMAN PLASMA OR SERUM

By NATHAN LIFSON

(From the Department of Physiology, University of Minnesota Medical School, Minneapolis)

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The purpose of this communication is to present data obtained by the thermoelectric vapor tension method of Hill and Baldes on the following aspects of the total osmotic activity of the plasma or serum of normal humans: (a) variability in the same individual in the same day, (b) variability in the same individuals from day to day, (c) effect of complete deprivation of both food and water. The information on these points in the literature appears to be, at best, very fragmentary.

Methods

The subjects were apparently healthy white males, aged 20 to 35 years. Blood was taken by syringe and needle from the antecubital vein of the seated subject. The latter compressed the upper arm with his free hand until the vein was entered, the pressure lasting perhaps 5 seconds. After a delay of an additional 2 to 5 seconds, from 4 to 7 cc. of blood were drawn under mineral oil. No muscular movements of the arm from which the blood was taken were permitted. Subsequent procedure depended upon whether plasma or serum was to be obtained. When plasma was desired, the blood was transferred immediately under mineral oil to centrifuge tubes containing 2 to 3 cc. of oil plus not more than 1 mg. of dry heparin (Connaught). Frequently the air space above the oil was replaced by alveolar air. After being corked, the tubes were placed in the refrigerator for from a few moments to 2 to 3 hours, whereupon they were centrifuged for 15 to 30 minutes and the plasma removed preparatory to determination of osmotic activity. When serum was desired, the procedure differed only in that heparin was omitted and coagulation was allowed to proceed in the refrigerator for at least 30 minutes. The samples were stored in the refrigerator except when removed for analysis. Preliminary experiments indicated that serum and heparinized plasma may be considered as equivalent in osmotic activity, and that refrigerator storage for as long as 7 days did not significantly change the osmotic activity of the serum or plasma.

Osmotic activity was measured by the thermoelectric vapor tension method of Hill and Baldes (1, 2) at a temperature of 37.5° and in a gas phase of 5 per cent CO₂ in oxygen. In all but a very few instances, the analyses

were performed in duplicate or triplicate. The results are expressed in terms of the concentration in milliequivalents per kilo of water of an isosmotic NaCl solution. The reference solution, the solution on the walls of the thermocouple chamber, and the unknown sample were always within 8 milliequivalents per kilo of water of one another in osmotic activity. A difference between samples of more than 1.2 milliequivalents per kilo is of statistical significance (3). No correction was made for the osmotic activity of the CO₂ which dissolves in the reference NaCl solution; this amounts to approximately 0.6 milliequivalent per kilo.

The experiments were carried out between May 27, 1942, and August 5, 1942. During this period, according to the data for Minneapolis of the

TABLE I

Variability of Osmotic Activity of Serum or Plasma in Same Individual within Same Day

The values are given in terms of milliequivalents of NaCl per kilo of water. Sample Pairs 6 to 9 were obtained under conditions of complete fast.

Sample pair No.	Subject	Osmotic activity		
		A.m.	P.m.	Change (p.m. - a.m.)
1	I. A.	152.4	153.1	+0.7
2	F. K.	157.3	158.2	+0.9
3	W. K.	156.7	156.6	-0.1
4	V. L.	153.3	153.0	-0.3
5	B. T.	153.3	153.0	-0.3
6	I. A.	153.8	152.3	-1.5
7	F. K.	153.0	155.0	+2.0
8	W. K.	152.6	153.4	+0.8
9	V. L.	154.4	153.4	-1.0

United States Weather Bureau, the mean daily temperature ranged from 12-31°; the relative humidity readings ranged from 32 to 99 per cent; and the wind velocity averaged 9 to 10 miles per hour.

Results

Variability of Osmotic Activity of Serum or Plasma in Same Individual within Same Day—The data have been listed in Table I. Sample Pairs 1 to 5 were obtained without any restrictions imposed upon the subjects, except that no unusual behavior (food and water intake (4, 5), muscular exertion (5), etc.) was indulged in. The differences between morning and afternoon samples under these conditions were all less than 1.0 milliequivalent per kilo of water. The values for sample Pairs 6 to 9 were obtained with the subjects refraining from taking food or drink during the experi-

mental period. The variations are noted to be 0.8 to 2.0 milliequivalents per kilo, but with no constancy in the direction of the change.

Day to Day Variations in Osmotic Activity of Serum or Plasma—The data have been listed in Table II, wherein it will be noted that for the seven subjects studied over a period of 8 to 57 days, the range of individual variation in osmotic activity of serum or plasma was from 1.6 to 5.2 milliequivalents per kilo. A study of the sequence of the values did not reveal any obvious reason for the observed variations.

Effect of Deprivation of Both Food and Drink—Under the conditions of our experiments, no significant change in osmotic activity of serum or plasma occurred before 24 hours of complete fast had elapsed. One 48 hour complete fast was conducted in August, 1942, during which there prevailed

TABLE II
Day to Day Variations in Osmotic Activity of Serum or Plasma
The values are given in milliequivalents of NaCl per kilo of water.

Subject	Period covered	No. of days sampled	No. of samples	Osmotic activity	
				Absolute range	Differential range
	<i>days</i>				
I. A.	57	6	8	152.3-155.9	3.6
A. G.	35	3	3	152.5-156.2	3.7
F. K.	14	3	5	153.0-158.2	5.2
W. K.	14	5	7	152.4-156.7	4.3
N. L.	8	4	4	154.6-156.2	1.6
V. L.	13	5	7	153.0-156.5	3.5
B. T.	16	5	6	151.1-154.0	2.9

an environmental temperature range of 18-29°, and a relative humidity range of 47 to 94 per cent. Control determinations of the serum osmotic activity on four different days displayed a range of variation of 1.6 milliequivalents per kilo. After 40 hours of complete fast, the serum osmotic activity had risen 1.3 milliequivalents per kilo above the mean of the control values; and after 48 hours, it had increased an additional 1.2 milliequivalents per kilo. At this time the subject had lost 3.4 per cent of his body weight. 16 hours after termination of the fast, the osmotic activity of the serum had fallen 4.5 milliequivalents per kilo to a value 2 milliequivalents per kilo below the mean of the control values. However, no single observation was outside the range which was noted for normals (see below).

Total Osmotic Activity of Normal Human Blood—In Table III have been placed the characteristics of the distributions of osmotic activity for normal blood as determined by the thermoelectric vapor tension method. The

mean for our series of twenty-eight subjects was 155.5 milliequivalents per kilo, with a standard deviation of the distribution of 1.9 milliequivalents per kilo, and a range from 152 to 159 milliequivalents per kilo. When several values were available for a single subject, these were averaged, so that each individual was represented only once in this series.

The means reported by Culbert (6), by Benham *et al.* (7),¹ and that of the present series lie between 154 and 156 milliequivalents per kilo, and the standard deviations of the distributions vary from 1.9 to 3.1 milliequivalents per kilo. These mean values are about 6 milliequivalents per kilo lower than the mean (161.6 ± 1.2 milliequivalents per kilo) reported by Margaria for adult males (5). It is concluded that the values

TABLE III

Comparison of Distributions Presented by Various Investigators for Osmotic Activity of Normal Human "Blood" As Determined by Thermoelectric Vapor Tension Method

The values are given in milliequivalents of NaCl per kilo of water.

Investigator	Method	Sample	Location	Subjects	Mean	σ distribution	Range
Margaria	Hill	Defibrinated blood	England	19 adult males	161.6	1.2	159-164
				16 adult females	158.6	1.5	155-160
Culbert	"	" "	New York	30 boys	154.1	2.9	148-160
				20 girls	154.9	2.9	146-159
Benham <i>et al.</i>	Baldes	Serum	England	21 adults	156.0	3.1	151-163
Present data	"	" or plasma	Minneapolis	28 adult males	155.5	1.9	152-159

reported by Margaria are probably erroneously high, as suggested earlier by Baldes, who also states that, "Undoubtedly, the measurements [of Margaria] were not as accurate as some made subsequently . . ." (8).

SUMMARY

For the total osmotic activity of the serum or plasma of normal adult human males, it was found that (a) the variation from morning to afternoon did not exceed 2.0 milliequivalents per kilo of water; (b) the variation from

¹ The results for the glaucomatous and non-glaucomatous subjects of Benham *et al.* have been combined. Since there was no significant difference in osmotic activity of the serum between these two groups of patients with diseases of the eye, they have been considered as "normal" for purposes of the present discussion.

day to day in the same individual ranged from 1.6 to 5.2 milliequivalents per kilo; (c) deprivation of both food and water for 24 hours produced no significant change under the conditions of these experiments. The results of one 48 hour period are described.

For twenty-eight subjects, the mean total osmotic activity of the serum or plasma of normal adult human males was found to be 155.5 milliequivalents per kilo, with a range of 152 to 159 milliequivalents per kilo, and a standard deviation of the distribution of 1.9 milliequivalents per kilo.

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INFLUENCE OF SULFANILAMIDE ON THE GERMINATION OF SEEDS

By FONSECA RIBEIRO

(From the Department of Biological Chemistry, University of São Paulo,
São Paulo, Brazil)

(Received for publication, November 1, 1943)

Sulfanilamide is a therapeutic agent with the property of acting upon certain bacterial cells, considered as plant cells; therefore it should be reasonable to inquire as to the possible effect of this drug upon higher organisms of the same kingdom. It is presumable that, if a plant could be influenced by sulfanilamide, such influence should have greater possibility of occurring in more sensitive organs or in more delicate stages of plant growth; hence we decided to use germinating seeds as experimental material. Following some preliminary tests we chose rice seeds (*Oryza sativa*), in view, above all, of their rapid development. In fact, when placed in a sufficient degree of humidity, such seeds show beginning development at the end of 4 to 5 days and then, after 3 or 4 more days, they attain considerable dimensions of stem as well as of the principal root (3.5 to 5 cm.). It must be said, however, that several other seeds experimented with behaved similarly to rice in regard to the influence of sulfanilamide.

The germination of the seeds was always carried out on Petri dishes in which a thick disk of filter paper was placed; thus 10 cc. of liquid (water or the solution to be examined) could be added to provide the necessary conditions of humidity for germination.

Sulfanilamide, starting with none and increasing by 1 mg., was poured into a series of eleven Petri plates, each containing 10 cc. The concentrations of the drug therefore varied as 1:10,000, 2:10,000, 3:10,000, etc. 50 seeds were put in each plate and the plates left at room temperature, under a glass bowl, to avoid evaporation.

At the end of 5 days the development of the seeds in Plate 1 (control) was evident, as may be seen in Fig. 1; the sprouts varied in size from 3 to 6 mm. and the roots from 20 to 30 mm. In Plate 2 (1 mg. of sulfanilamide) only 40 per cent of the seeds showed roots of appreciable development, but to a lesser degree than those in Plate 1. In Plate 3 (2 mg. of sulfanilamide in 10 cc.) only 18 per cent of the seeds had more or less developed roots, and in Plate 4, but 8 per cent. In all the other plates the aspect was the same (Fig. 2): the sprouts were of normal appearance but not above 1 to 3 mm. in length and the roots were atrophied in every case, none being more than 1 to 4 mm.

In the following days there was no change in aspect: the effect of the drug upon the growth of the embryo of the seeds was already evident in the



FIG. 1



FIG. 2

FIG. 1. Normal development of rice seeds sown in 10 cc. of distilled water (5th day of development).

FIG. 2. Rice seeds sown in 10 cc. of sulfanilamide solution in distilled water at 1:2000 (5th day of development).

TABLE I

Antagonism between Sulfanilamide and p-Aminobenzoic Acid in Germination of Rice Seeds

Plate No	Sulfanilamide, 1 per cent	<i>p</i> -Aminobenzoic acid, 1 per cent	Distilled water	Development of seeds
	cc	cc	cc	
1	0	0	10	++++
2	5	0	5	—
3	0	5	5	++++
4	1	1	8	++++
5	3	1	6	+++
6	5	1	4	+
7	5	2	3	++
8	5	3	2	++++
9	5	4	1	++++
10	5	5	0	++++

1:10,000 solution, but more marked in the 1:2000 solution. Even this last solution does not, however, kill the embryo, which for many days continues to have normal aspect and turgescence.

In order to judge the behavior of seeds under the influence of drugs of

different type of action from that of sulfanilamide, we carried out similar tests, employing phenol and arsenobenzene in solutions at 1:10,000. The seeds in the plates containing phenol were entirely sterile with no evidence of germination, and in those containing arsenobenzene about 80 per cent was without germination, whereas in the remaining seeds the growth was only retarded, the roots attaining lengths not very different from those of the seeds in the control plate.

These results are in accordance with findings on microbial agents. While sulfanilamide shows a bacteriostatic action, phenol is a microbicide and arsenobenzene, which is a specific agent for certain microorganisms (spirochetes), has a lethal effect on the germination of the seeds.

We tried then to investigate the antagonism existing between sulfanilamide and *p*-aminobenzoic acid. In Table I there are indicated the conditions of the experiment and the results observed. From these results it is evident that the antagonism of *p*-aminobenzoic acid, in relation to sulfanilamide, is the same as in cultures of bacteria.

. It seems reasonable to us that this technique for investigating the action of sulfanilamide may permit attacking problems of which the solution would not be possible by experiments *in vivo*, or even in microbial cultures, as for example, facilitating the study of the mechanism of action of the drug.

A RAPID SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF METHEMOGLOBIN AND CARBONYLHEMOGLOBIN IN BLOOD

BY B. L. HORECKER AND F. S. BRACKETT

(From the Division of Industrial Hygiene, National Institute of Health,
Bethesda, Maryland)

(Received for publication, August 18, 1943)

Little use has thus far been made of the near infra-red spectrum for the identification and determination of hemoglobin and its derivatives. By means of infra-red photography Eggert (1) established the transparency of carbon monoxide-saturated blood and used this property for the qualitative detection of carbon monoxide poisoning. Eggert's observations were extended and placed on a more quantitative basis by Merkelbach (2). A further advance was made by Matthes and Gross (3) who developed a method for the determination of the carbon monoxide content of blood *in vivo* in which the absorption of red and infra-red radiation by flowing blood was measured in the ear lobe. A band for reduced hemoglobin at 7550 Å has been described by Sidwell, Munch, Barron, and Hogness (4) and employed by these workers for the spectrophotometric determination of the degree of oxygenation of hemoglobin solutions.

In the near infra-red region of the spectrum, between 7000 and 10,000 Å, the hemoglobin derivatives have absorption bands which, because of their lower specific absorption, become apparent only when solutions more concentrated than those generally employed for spectrophotometric work are examined. A detailed description of these bands is contained in a previous publication (5). Despite the lower absorption, the large differences between the spectra of methemoglobin and cyanmethemoglobin and between oxyhemoglobin and carbonylhemoglobin make this region well suited for analytical purposes. On the basis of these considerations we have developed a spectrophotometric method for the determination of methemoglobin and carbonylhemoglobin in blood. The method is rapid and accurate and requires only a few manipulations. It has an advantage over existing methods in that it permits the determination of the total hemoglobin, carbonylhemoglobin, and methemoglobin content in a single blood sample.

Principle of Method

For each sample of blood to be analyzed, three absorption measurements are made. From the first of these measurements, in the infra-red, a density D_1 is obtained which may be expressed as follows:

$$(1) \quad D_1 = (\epsilon_{\text{HbO}_2}^1 C_{\text{HbO}_2} + \epsilon_{\text{MHB}}^1 C_{\text{MHB}} + \epsilon_{\text{HbCO}}^1 C_{\text{HbCO}}) \frac{L}{d_1}^*$$

where $\epsilon_{\text{HbO}_2}^1$, ϵ_{MHB}^1 , and ϵ_{HbCO}^1 are the extinction constants for the wave-length interval used and C_{HbO_2} , C_{MHB} , and C_{HbCO} are the concentrations in the original blood sample of oxyhemoglobin, methemoglobin, and carbonylhemoglobin, respectively, d_1 is the dilution factor for the sample measured, and L is the length of the light path through the absorption cell.

The sample in the absorption cell is then treated with KCN to convert the methemoglobin present to cyanmethemoglobin, and the density measured at the same wave-length interval.

$$(2) \quad D_1 = (\epsilon_{\text{HbO}_2}^1 C_{\text{HbO}_2} + \epsilon_{\text{MHbCN}}^1 C_{\text{MHB}} + \epsilon_{\text{HbCO}}^1 C_{\text{HbCO}}) \frac{L}{d_1}$$

where $\epsilon_{\text{MHbCN}}^1$ is the extinction constant for cyanmethemoglobin at the wave-length used. From these two equations the concentration of methemoglobin in the blood sample may be computed.

Essentially the same principle has been employed by Evelyn and Malloy (6) for the photoelectric determination of methemoglobin, with a spectral band in the vicinity of 6350 Å. The infra-red region is to be preferred because the absorption bands are relatively broad and carbonylhemoglobin and cyanmethemoglobin are more transparent.

The sample is then diluted and a third determination made in the visible region.

$$(3) \quad D_2 = (\epsilon_{\text{HbO}_2}^2 C_{\text{HbO}_2} + \epsilon_{\text{MHbCN}}^2 C_{\text{MHB}} + \epsilon_{\text{HbCO}}^2 C_{\text{HbCO}}) \frac{L}{d_2}$$

where $\epsilon_{\text{HbO}_2}^2$, $\epsilon_{\text{MHbCN}}^2$, and ϵ_{HbCO}^2 are the respective extinction constants for the second spectral interval and d_2 is the second dilution factor. Since C_{MHB} is already known, a solution of Equations 1 and 3 will yield the values of C_{HbO_2} and C_{HbCO} .

Instruments—The method has been applied to the Coleman spectrophotometer, model 10S, with a 5 mμ slit, and to several portable photometers especially designed for this purpose. These photometers are alike in general design but differ somewhat in optical features. In every case the light beam is split into two parts each of which ultimately activates one of

* The molecular extinction coefficient ϵ is defined by the equation $D = \log_{10} I_0/I = \epsilon CL$ where C is the concentration in equivalents per liter and L the cell length in cm. This departs somewhat from the notation used in a previous publication (5) in which the absorption coefficient α was used to designate the coefficients obtained when C was expressed in equivalents per cc. It would appear to be more consistent with general practice to reserve the absorption coefficient α for constants defined in terms of natural logarithms, $\log_e I_0/I = \alpha CL$.

a pair of matched photocells which are mounted in a balanced electrical circuit according to Hanson (7). One light beam passes through the absorption cell containing the sample. The second beam traverses some arrangement for reducing the light intensity to equal that transmitted by the absorption cell. This consists of either a neutral wedge of density 1.0 attached to a vernier scale, or a spiral disk designed to reduce the aperture of a diaphragm linearly with rotation of the disk. The results described below were obtained with a photometer containing the neutral wedge; similar results have also been obtained with other models in which the spiral disk is employed.

Selection of Wave-Lengths—Absorption measurements on the Coleman spectrophotometer were made at dial settings of 4965 and 8000 Å. 8000 Å is sufficiently close to the maximum for alkaline methemoglobin to afford a high sensitivity and in addition is isobestic for oxyhemoglobin and reduced hemoglobin; the presence of reduced hemoglobin will therefore not interfere with the determination. For these reasons 8000 Å is to be preferred to the absorption maximum for oxyhemoglobin at 9200 Å, which would be somewhat more sensitive for the carbonylhemoglobin determination.

The spectral interval at 4965 Å constitutes an isobestic point for oxyhemoglobin and carbonylhemoglobin, so that loss of CO during the final dilution will not affect the results. At each of the spectral intervals selected the absorption curves for all the components concerned are relatively flat, insuring a minimum of deviation from Beer's law.

In the selection of filters for the photometers various combinations were tested in a search for spectral intervals isobestic for oxyhemoglobin and carbonylhemoglobin in the visible and for oxyhemoglobin and reduced hemoglobin in the infra-red region. Because the effective spectral interval is in part a function of the phototube and lamp characteristics, different combinations must be tested in every case. The particular photometer used in this study, equipped with RCA No. 919 and No. 917 phototubes, contained the following Corning filters: Set 1, No. 3385, 1.1 mm.; No. 5030, 6.0 mm.; No. 255, 2.0 mm.; Set 2, No. 338, 4.0 mm.; No. 4303, 5.1 mm.; No. 5543, 2.6 mm. Set 1 isolates a suitable band in the infra-red region in the vicinity of 8000 Å and Set 2 a suitable band in the visible region near 5000 Å. Although oxyhemoglobin and carbonylhemoglobin are not perfectly identical in absorption in the region isolated by Set 2, they are sufficiently alike for the purposes of the method.

Analytical Procedure

Solutions—Since the absorption spectrum of methemoglobin is dependent upon the pH of the solution, it is necessary to work with buffered solutions. We have found it most convenient to use borate buffer at pH 9.4. 24.8 gm.

of H_3BO_3 were dissolved in 0.20 N NaOH to a final volume of 1 liter; 80 cc. of this solution were then mixed with 20 cc. of 0.20 N NaOH to form the stock 0.2 N buffer.

A hemolyzing solution was prepared by dissolving 0.3 gm. of saponin (Eimer and Amend, pure white grade) in 10 cc. of the 0.2 N borate buffer and diluting to 100 cc. This hemolyzing solution was freshly prepared each morning, since it becomes turbid on standing.

Absorption Measurements—Blood samples were collected by venipuncture and treated with approximately 0.01 volume of 25 per cent potassium oxalate. For the absorption measurements, the blood sample was accurately diluted 1:10 with the hemolyzing solution and allowed to stand a minute or two to insure complete hemolysis. The solution was then transferred to the absorption cell and the transmission (T_1) measured at 8000 Å on the Coleman spectrophotometer and the density (D_1) with filter Set 1 on the photometer. The solution in the cell was then treated with a few crystals of KCN, stirred, and the transmission (T_2) and density (D_2) again measured on each instrument at the same wave-length and filter settings. Finally, 1 cc. was withdrawn from the absorption cell, diluted accurately to 25 cc. with water, and the transmission (T_3) of this solution measured at 4965 Å on the Coleman spectrophotometer and the density (D_3) with filter Set 2 on the photometer.

Calibration of Instruments—For the computation of results from Equations 1 to 3, it is necessary to know the molecular extinction constants for each hemoglobin derivative at each spectral interval. These were determined with samples of normal blood from non-smoking individuals, the total hemoglobin content of which was determined independently by spectrophotometric analyses. Each sample was divided into three portions: one portion was completely oxygenated, a second was saturated with CO, and the third converted to methemoglobin by the addition of $\text{K}_3\text{Fe}(\text{CN})_6$ (30 mg. per cc. of blood). Each of these samples was then analyzed in the manner described above. For the absorption measurements on the methemoglobin solutions the comparison cells were filled with solutions containing equivalent concentrations of $\text{K}_3\text{Fe}(\text{CN})_6$. The transmission values obtained from the Coleman spectrophotometer (T_1 , T_2 , and T_3) were converted to density units (D_1 , D_2 , and D_3) by the following equation.

$$D = \log \frac{1}{T}$$

From the calibration samples the extinction constants were computed by applying the relation

$$D = \epsilon CL$$

where ϵ is the molecular extinction constant for the particular spectral region and derivative employed, C is the concentration in equivalents per liter on an Fe basis, and L the length of the absorption cell in cm.

In Table I are listed the extinction constants obtained for each of these instruments.

The constants obtained with the Coleman spectrophotometer, because of the relatively narrow effective slit width used, are similar to those previously published for precise spectrophotometric work. The constants obtained with the photometer, however, differ widely from those obtained with monochromatic light, because of the difficulty of obtaining narrow intervals by means of filters.

TABLE I
Extinction Constants of Hemoglobin Derivatives

Filter region	Instrument	Extinction constants, $\epsilon \times 10^{-3}$ $C = 1$ equivalent per liter, $L = 1$ cm.			
		HbO ₂	HbCO	MHb	MHbCN
Visible	Coleman	5.50	5.69		7.27
	Photometer	6.59	6.93		8.78
Infra-red	Coleman	0.235	0.055	0.525	0.069
	Photometer	0.252	0.050	0.421	0.064

From Equations 1 to 3 the following equations may be developed for the computation of the results.

$$(4) \quad C_{\text{MHb}} = \frac{(D_1 - D_2)d_1}{(\epsilon_{\text{MHb}}^1 - \epsilon_{\text{MHbCN}}^1)L}$$

$$(5) \quad R = \frac{D_1 - \frac{L}{d_1} C_{\text{MHb}} \epsilon_{\text{MHb}}^1}{D_2 - \frac{L}{d_2} C_{\text{MHb}} \epsilon_{\text{MHbCN}}^2}$$

$$(6) \quad f_{\text{HbCO}} = \frac{R_{\text{HbO}_2} - R}{R_{\text{HbO}_2} - R_{\text{HbCO}}}$$

$$(7) \quad C_{\text{HbO}_2} + C_{\text{HbCO}} = \frac{d_2 \left(\frac{D_2 - \frac{L}{d_1} C_{\text{MHb}} \epsilon_{\text{MHbCN}}^2}{\epsilon_{\text{HbO}_2}^2 + (\epsilon_{\text{HbCO}}^2 - \epsilon_{\text{HbO}_2}^2) f_{\text{HbCO}}} \right)}{L}$$

$$(8) \quad C_{\text{total}} = C_{\text{MHb}} + (C_{\text{HbO}_2} + C_{\text{HbCO}})$$

$$(9) \quad C_{\text{HbCO}} = f_{\text{HbCO}}(C_{\text{HbO}_2} + C_{\text{HbCO}})$$

where C_{MHb} , C_{HbO_2} , and C_{total} are the respective concentrations expressed in equivalents per liter, d_1 and d_2 are the dilution factors, L is the length of

the cell, and R is the ratio of the infra-red to the visible absorption, after these have been corrected for the absorption due to methemoglobin. R_{HbO_2} and R_{HbCO} are the ratios for solutions containing these derivatives only and may be computed from the calibration constants. f_{HbCO} is the fraction of ferrous hemoglobin present as HbCO. The dilution factors d_1 and d_2 were equal to 10 and 250 respectively. The cell lengths L were 1.00 cm. for the wedge photometer and 1.30 cm. for the Coleman spectrophotometer.

Substitution of the appropriate values for ϵ from Table I in Equations 4 to 7 yields the following equations for the particular instruments used in these studies.

Coleman Spectrophotometer

$$(4, a) \quad C_{\text{MHB}} = (D_1 - D_2)16.9 \times 10^{-3}$$

$$(5, a) \quad \begin{array}{l} D_1 - 68.2C_{\text{MHB}} \\ R_{\text{obs.}} \quad D_2 - 37.8C_{\text{MHB}} \end{array}$$

$$(6, a) \quad f_{\text{HbCO}} = \frac{1.070 - R_{\text{obs.}}}{0.830}$$

$$(7, a) \quad C_{\text{HbO}_2} + C_{\text{HbCO}} = \frac{(D_2 - 37.8C_{\text{MHB}})250 \times 10^{-3}}{7.15 + 0.25f_{\text{HbCO}}}$$

Photometer

$$(4, b) \quad C_{\text{MHB}} = (D_1 - D_2)28.1 \times 10^{-3}$$

$$(5, b) \quad \begin{array}{l} D_1 - 42C_{\text{MHB}} \\ R \quad D_2 - 35C_{\text{MHB}} \end{array}$$

$$(6, b) \quad f_{\text{HbCO}} = \frac{0.954 - R}{0.776}$$

$$(7, b) \quad C_{\text{HbO}_2} + C_{\text{HbCO}} = \frac{(D_2 - 35C_{\text{MHB}})250 \times 10^{-3}}{6.59 + 0.34f_{\text{HbCO}}}$$

When the corrections for the small differences in absorption between HbCO and HbO₂ in the visible regions are neglected, Equations 7, *a* and 7, *b* reduce to

$$(7, a') \quad C_{\text{HbO}_2} + C_{\text{HbCO}} = (D_2 - 37.8C_{\text{MHB}})35.0 \times 10^{-3}$$

$$(7, b') \quad C_{\text{HbO}_2} + C_{\text{HbCO}} = (D_2 - 35C_{\text{MHB}})37.9 \times 10^{-3}$$

The error introduced by this simplification is negligible for sublethal concentrations of HbCO.

The computation of results may be greatly simplified by the use of suitable nomograms in place of the equations presented above. Such nomograms have been prepared for the Coleman spectrophotometer and the wedge photometer used in these studies.

Results

A number of samples of human blood were treated with varying amounts of NaNO_2 and pure CO to bring about partial conversion of these samples to methemoglobin and carbonylhemoglobin. For the same samples the CO content was determined by the method of Horvath and Roughton (8) and the total and active hemoglobin contents were determined according to the procedures described in Peters and Van Slyke (9). In Table II we compare the results of the spectrophotometric analyses with those obtained by the gasometric methods. The nomograms referred to above were used

TABLE II

Comparison of Blood Analyses on Coleman Spectrophotometer and Neutral Wedge Photometer with Van Slyke Analyses

The values are given in milliequivalents per liter.

Sample No.	Total hemoglobin			Methemoglobin			Carbonylhemoglobin		
	Coleman	Photometer	Van Slyke	Coleman	Photometer	Van Slyke	Coleman	Photometer	Van Slyke
1	8.41	8.29	8.41	1.44	1.32	1.32	2.29	2.15	2.22
2	8.40	8.37	8.41	1.22	1.21	1.24	1.79	2.05	2.23
3	9.72	9.62	9.73	0.52	0.53	0.51	1.96	1.80	1.87
4	9.66	9.77	9.73	0.39	0.43	0.39	1.75	2.05	1.80
5	10.18	10.27	10.27	0.08	0.15	0.01	3.25	3.20	3.14
6	10.24	10.27	10.27	1.42	1.41	1.41	3.20	3.00	3.09
7	10.27	10.31	10.27	0.95	1.07	1.05	3.16	3.10	3.11
8	9.24	9.37	9.34	0.18	0.23	0.10	1.75	1.85	2.07
9	9.33	9.22	9.29	4.09	4.03	3.62	2.08	1.80	1.98
10	9.44	9.43	9.29	3.89	3.74	3.34	2.00	1.85	2.03
11	9.46	9.43	9.44	1.28	1.26	1.16	0.29	0.05	0.09
12	9.46	9.43	9.41	0.58	0.59	0.61	1.12	1.15	1.25
13	9.38	9.44	9.41	0.42	0.48	0.33	1.29	1.40	1.38
14	9.24	9.18	9.13	0.90	0.93	0.98	0.37	0.25	0.17
15	9.23	9.27	9.13	0.64	0.63	0.38	1.75	1.65	1.64

for the computation of the spectrophotometric data; essentially the same values are given when the equations are used.

The concentrations of the hemoglobin components in Table II are given in terms of milliequivalents per liter. These may be converted to gm. per 100 cc. by multiplying by 1.67, and to volumes per cent by multiplying by 2.24.

DISCUSSION

An examination of Table II discloses that the results of spectrophotometric analysis compare very favorably with results obtained with the

Van Slyke apparatus. Standard deviations from the Van Slyke data have been computed and turn out to be 0.07 for total hemoglobin, 0.10 for methemoglobin, and 0.14 for carbonylhemoglobin, for both of the instruments used. The accuracy is thus about 0.7 per cent for total hemoglobin, 0.9 per cent for methemoglobin, and 1.5 per cent for carbonylhemoglobin. Attention is drawn to the unusually large discrepancies observed in the MHb determinations in Samples 9 and 10. These results as well as the carbonylhemoglobin determination on the Coleman spectrophotometer for Sample 2 have been omitted from the computation of standard deviations. The discrepancies in the methemoglobin determinations are undoubtedly due to a failure of the standard Van Slyke method for total hemoglobin with samples containing high concentrations of MHb. The reproducibility of the Van Slyke method for such samples is very poor, and may be attributed to a failure to obtain complete reduction of the MHb with $\text{Na}_2\text{S}_2\text{O}_4$. It will be observed that the results obtained from the two photoelectric instruments are in good agreement.

In the visible region the absorption contributed by blood components other than hemoglobin is negligible (5). In the infra-red region, however, an appreciable contribution is made by absorption and scattering from material in the blood plasma and from red cell fragments. If normal blood is treated uniformly, this contribution is sufficiently constant to permit accurate determinations. It has been observed, however, that the infra-red absorption is influenced by the manner in which the blood is collected. Thus, solutions prepared by immediately hemolyzing freshly drawn blood without the addition of anticoagulant will be appreciably more transparent than solutions treated with oxalate in the manner described above. Any convenient method may be used, provided the calibration constants are determined in the same way and the same procedure is followed throughout. While the presence of unusually large or small amounts of scattering or absorbing material has little effect on the determination of methemoglobin and total hemoglobin, the CO determination is more sensitive and may be in error by substantial amounts. For this determination particular care must be exercised. It is recommended that no samples be taken for several hours after a moderately heavy meal. The results described in Table II were obtained with samples taken in the forenoon, the subjects having consumed an unrestricted breakfast.

If analyses of the methemoglobin content alone are desired, the method may be simplified by eliminating the determination in the visible region. In such case the total hemoglobin may be estimated from the absorption of the original solution, with an accuracy which will depend on the CO content of the blood.

Scholander and Roughton (10) have recently described a micro gaso-

metric method for the determination of CO in blood which is simple and accurate and suitable for field use. It has important advantages over the present method in that no photometer is required and smaller blood samples can be analyzed. It does, however, require a considerable number of reagents, whereas the spectrophotometric method requires but one readily prepared solution. The present method can be used for the simultaneous estimation of total hemoglobin and methemoglobin, as well as carboxyhemoglobin, and is exceedingly simple and rapid. The method can readily be adapted to any photometer which extends into the near infrared region. In view of the wide spectral intervals isolated by the filter systems employed in the wedge photometer it is felt that the method can be successfully applied with sufficient accuracy to any spectrophotometer which isolates a band no broader than about 300 or 400 Å.

The authors are indebted to Dr. John S. Kirby-Smith for the construction of the photometer, to Dr. H. L. Andrews for helpful suggestions, and to Mr. William Pricer for the Van Slyke analyses.

SUMMARY

1. A simple spectrophotometric method for the simultaneous determination of methemoglobin, carboxyhemoglobin, and total hemoglobin is described.

2. Details of the method as applied to the Coleman spectrophotometer and to a special photometer constructed for this purpose are given.

3. Results obtained by this method are compared to Van Slyke gasometric determinations. The method is accurate to about 1 per cent.

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FERRITIN AND APOFERRITIN IN THE ULTRACENTRIFUGE

STUDIES ON THE RELATIONSHIP OF FERRITIN AND APOFERRITIN; PRECISION MEASUREMENTS OF THE RATES OF SEDIMENTATION OF APOFERRITIN

By ALEXANDRE ROTHEN

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

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Studies on Relationship of Ferritin and Apoferritin

Since the discovery by Laufberger (1) of a crystallizable protein from horse spleen containing over 20 per cent of iron, workers in two laboratories have studied this interesting material. Kuhn and coworkers (2) repeated Laufberger's experiments and considered this iron protein to be a well defined chemical entity containing 50 per cent of protein, 12 per cent of nucleic acid, and 35 per cent of iron hydroxide. Granick (3) and Granick and Michaelis (4), however, have made a thorough study of ferritin and were not able to confirm the presence of nucleic acid as claimed by Kuhn.

It was of interest to examine this unusual protein in the ultracentrifuge. It might have been expected that the beautifully crystalline material would exhibit, like most crystalline proteins, a single component in the ultracentrifuge and thus have a well defined molecular weight. Kuhn was so convinced of the homogeneity of ferritin that on the basis of the histidine content he advanced the view that the molecular weight of ferritin is a multiple of the still problematic Svedberg protein unit (5).

The present study has shown that crystalline ferritin is not a well defined molecule. The views of Kuhn concerning the structure of ferritin, including his contention that there is 1 iron atom for each peptide linkage, have been proved untenable.

The substances used in this study were kindly provided by Dr. Granick whose papers (3, 4) should be consulted for the method of preparation.

Ultracentrifugation of Ferritin—When a clear solution of ferritin, which has a deep brown color, is submitted to a high centrifugal field, say 150,000 times gravity, one observes that the colored material sediments rapidly, is very heterogeneous, and leaves behind it a colorless, more slowly moving substance, yielding a very sharp boundary, with a sedimentation constant of about 17 Svedberg units (6). Fig. 1 represents a tracing of a schlieren diagram obtained during such an experiment. It was taken 5 minutes after a maximum velocity of 43,200 R.P.M. (mean radius 6.5 cm.) was reached. This experiment demonstrates that ferritin is not a chemical entity but is a mixture of a colorless, homogeneous protein and a hetero-

geneous, colored material of large particle size. The next step was to determine the nature of these two components, to estimate the sizes of the particles and their relative amounts in the mixture. The methods used will be described later in the paper.

It has been reported by previous workers that the amount of iron in ferritin varies slightly according to the preparation. Granick has isolated ferritin from horse spleen with an iron content as low as 18 per cent and as high as 23 per cent (3). This fact alone is an indication of the heterogeneity of ferritin. It was found in this investigation, by analysis of the sedimentation patterns, that the colorless protein part amounted to 19 per cent in a preparation with 20.2 per cent iron, whereas in another sample containing 17.7 per cent iron about 26 per cent of the material consisted of the colorless protein. The variation in iron content arises, therefore, in

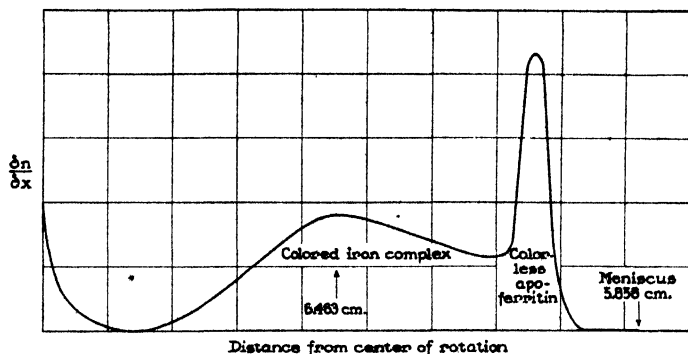


Fig. 1. Sedimentation pattern of horse ferritin

part at least, from the variable proportions of colorless protein in the mixture called ferritin.

An important contribution to the study of ferritin was made by Granick and Michaelis (4) when they found that they could remove the iron from ferritin by chemical means, without denaturing the protein. The latter, which they named apoferritin, could then be crystallized as easily as ferritin. Its crystals were similar to those of ferritin except that they were colorless, instead of deep brown. Apoferritin has been found to be a very homogeneous protein and was therefore selected for a study of the present accuracy of the ultracentrifugal method for determining molecular weights to be given in the second part of this paper. As will be seen, the molecular weight of apoferritin was calculated to be 470,000 from the sedimentation constant ($s'_{20} = 17.6$ Svedberg units), the diffusion constant, and the specific volume. The sedimenting boundary of apoferritin can be seen in Fig. 2, *a* and *b*, from which it appears that there was also present a small

amount of a heavier protein ($s \simeq 25$ Svedberg units) whose percentage varied slightly in the different samples tested. It amounted to 1 per cent in Fig. 2, *a* and 8 per cent in Fig. 2, *b*.

Since the free colorless component (Fig. 1) of ferritin was found to have the same sedimentation constant as apoferritin, 17 Svedberg units, the identity of the two components was tested by separating the colorless material in the centrifuge with the aid of separation cells. The separation cells were lucite sector-shaped cells of 4° aperture, 12 mm. thick, and provided with a perforated partition situated at one-third of their length from

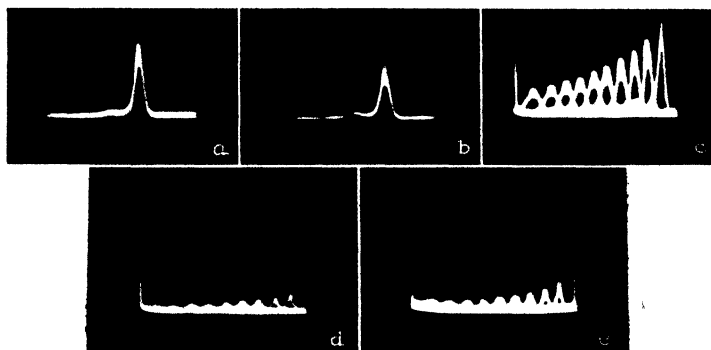


FIG. 2. Sedimentation patterns of horse apoferritin. (*a*) Experiment 1 of Table III, 23 minutes after reaching the maximum speed (43,200 R.P.M.); (*b*) Experiment 23 of Table III, 32 minutes after reaching the maximum speed (43,200 R.P.M.); (*c*) Experiment 23 of Table III. Ten successive exposures have been superimposed on the same plate. The time interval was 9 minutes with the exception of the interval between the fourth and the fifth exposures which was 10 minutes, and that between the fifth and the sixth which was 8 minutes; (*d*) Experiment 28 of Table III, velocity 43,200 R.P.M., nine superimposed exposures at 10 minute intervals; (*e*) Experiment 30 of Table III, velocity 54,000 R.P.M., nine superimposed exposures at 10 minute intervals. Note how much sharper the patterns are in this experiment at low temperature than those of (*d*) made at room temperature.

the bottom. The partition was covered with a piece of filter paper which prevented mixing when, to collect the solution from the upper and lower chambers, the rotor was removed from the shaft after an experiment. The separation was apparently as good as indicated by the schlieren pattern observed before the rotor came to rest. The protein thus isolated in pure form was crystallized by Granick and found to be identical with apoferritin. However, the question of the identity with apoferritin of the protein attached to the iron hydroxide remained. In this connection, Granick and Michaelis have been able, by chemical means, to recover at least 84 per cent of the nitrogen of ferritin as apoferritin nitrogen. As mentioned above, between 20 and 25 per cent only of *free* apoferritin is present in ferritin; so

that one may conclude therefrom that the major part of apoferritin is bound to iron hydroxide. Free apoferritin is not in an easily reversible equilibrium with the iron-bearing complex, as shown by the following experiment. The complex was separated from most of its free apoferritin by eleven successive centrifugal fractionations. From this isolated iron complex ultracentrifuge patterns demonstrated that no further apoferritin separated, at least during several following days.

As mentioned above, the ultracentrifuge studies show that the particles of the apoferritin-iron complex are not uniform. However, in spite of this heterogeneity the size of the average particle can be estimated from the average sedimentation rate. From the usual sedimentation equation it follows that if 2 molecules, *A* and *B*, have the same asymmetry factor, the following relation holds

$$\left(\frac{1 - V_B \rho}{1 - V_A \rho} \right) \frac{s_A}{s_B} = \left(\frac{M_A}{M_B} \right)^{\frac{1}{3}} \quad (1)$$

where *s*, *M*, and *V* are the sedimentation constant, the molecular weight, and the specific volume of the sedimenting molecules, respectively, and ρ is the density of the medium.

If it is assumed that apoferritin and the iron complex have the same asymmetry factor, it follows from the above equation, since $V_{\text{apof.}} = 0.747$, $V_{\text{complex}} = 0.55$ (this value was calculated from the experimental value $V_{\text{ferritin}} = 0.589$), $\rho \simeq 1$, $s_{\text{apof.}} = 18$ Svedberg units, $s_{\text{complex}} = 65$ Svedberg units, that the weight of the average iron complex is about 2.8 times that of apoferritin. Consequently, since there is about 38 per cent iron hydroxide in the iron complex, its particle contains on the average 2 molecules of apoferritin plus the iron hydroxide.

The lack of discontinuity in the widely spread boundary of the iron complex indicates that the size of the iron hydroxide unit is not uniform, and is on the average much smaller than the molecule of apoferritin. The available evidence therefrom is that iron atoms are not bound stoichiometrically to apoferritin, but are present as iron hydroxide micelles of various sizes combined with the apoferritin molecules.

Attempts by Michaelis, Coryell, and Granick (7) to make ferritin from apoferritin and iron hydroxide have been, however, unsuccessful. The preparations of colloidal ferric hydroxide they used were found to be polydisperse in the ultracentrifuge. The average sedimentation constant of the iron hydroxide micelle was 150 Svedberg units in one of their most finely dispersed preparations.¹ With the aid of Equation 1 this leads to the conclusion that the average iron hydroxide micelle was approximately

¹ This was the material referred to as Preparation T in the paper by Michaelis, Coryell, and Granick (7).

3 times the size of the apoferritin-iron complex. (The specific volume of iron hydroxide was assumed to be 0.260.) Thus the size of the iron hydroxide micelles probably prevented them from entering the molecular structure of apoferritin.

Two preliminary electrophoresis experiments were carried out with ferritin in a Tiselius apparatus at pH 4.5 (acetate buffer) and 7.5 (veronal buffer). At both pH values a single sharp boundary was observed. The protein thus moves with the same mobility whether or not it carries iron hydroxide micelles. Since electrophoretic mobilities depend upon surface charge densities, it would appear, therefore, that the iron hydroxide micelles do not affect the electrical state of the surface of apoferritin.

It is of interest to mention the fact that Kuhn (2) found that the optical activity of ferritin is of the order of magnitude of that of most proteins. This is also an indication that iron is not chemically bound to apoferritin. If it were bound, it might, indeed, have been expected that the strong absorption band in the violet region would be anisotropic and exhibit circular



FIG. 3. Sedimentation patterns of human apoferritin. (a) Experiment 31 of Table III, 28 minutes after reaching the maximum speed of 46,800; (b) Experiment 31 of Table III, seven successive pictures taken at 12 minute intervals.

dichroism with an anomalous rotatory dispersion in that band (Cotton effect). Consequently the optical rotation in the yellow part of the spectrum would be influenced appreciably. It is planned to study the rotatory dispersion of ferritin and apoferritin to make this point certain.

It was of interest to examine ferritin from other animals than the horse to discover whether species differences could be detected. Materials from man and dog were studied. On account of the polydisperse nature of ferritin, the rates of sedimentation of the apoferritin were studied.

It is apparent from Column 7 of Table III that the constants of sedimentation of human, horse, and dog apoferritin are very nearly the same. The same value is obtained for human and horse apoferritin ($s_{20}^0 = 17.6$ Svedberg units extrapolated to zero concentration), whereas the constant of dog apoferritin is 3 per cent greater ($s_{20}^0 = 18.1$ Svedberg units). Schlieren patterns of sedimenting human apoferritin can be seen in Fig. 3, *a* and *b*. It is observed that human apoferritin as well as horse apoferritin (see Fig. 2, *a* and *b*) contains a small percentage of a larger protein with $s \approx 25$ Svedberg units.

It has often been reported (8) that a large protein with a constant of sedimentation of 18 to 19 Svedberg units (a value very close to that found for apoferritin) is present in very small amounts in human and in horse sera. The possibility was then open that apoferritin and this large protein might be the same material. If they were, it meant that apoferritin, an iron carrier, was free in the blood stream. A horse globulin fraction was therefore prepared by Granick and submitted to eight successive fractionations in the ultracentrifuge. The final product contained over 90 per cent of the heavy component whose sedimentation constant was $s_{20}^{\circ} = 19.6$ Svedberg units, a value significantly higher than that found for apoferritin (see Fig. 4). Hence the two proteins are not the same. Granick was unable to crystallize this material and, furthermore, found that it did not give any precipitin reaction with anti-horse apoferritin immune serum. Although the constant of sedimentation of this heavy globulin was only slightly larger than that of apoferritin, the shapes of the molecules of these two proteins



FIG. 4. (a) sedimentation pattern of heavy horse globulin fraction obtained in a separation cell after eight successive fractional ultracentrifugations. A small amount of residual globulin can be seen to the right of the main component with $s \approx 19$ Svedberg units. The discontinuity observed in the base-line to the left is due to the partition in the cell. (b) Experiment 39 of Table III, seven pictures at successive intervals, from left to right, of 12, 14, 10, 12, and 12 minutes.

were found to be strikingly different. The diffusion constant of the heavy globulin (as reported by Svedberg) is $D_{20}^{\circ} = 1.8 \times 10^{-7}$, whereas that of apoferritin is twice as large, $D_{20}^{\circ} = 3.7 \times 10^{-7}$, with a molecular weight of about 470,000. The calculated asymmetry factor f/f_0 is 1.8 (heavy globulin) and 1.14 (apoferritin). This shows that the heavy globulin molecule is extremely asymmetric with an axis ratio of the order of 1:15.

Determination of Amount of Free Apoferritin in Ferritin—The amount of free apoferritin present in ferritin was determined by two methods which gave identical results. The first method consists in measuring the area under the curve of the sedimentation boundary ($\partial n/\partial x$ against x), which is proportional to the concentration. Experiments made with pure apoferritin showed that, with the optical system in use, a 1 per cent solution in a 12 mm. cell gave, for a slit angle of 35° , an area of 0.41 sq. cm. after correction for decrease in concentration during the run. In many cases this value remained remarkably constant whether the boundary was near the meniscus or near the bottom of the cell. In a few cases, however, a de-

crease in area (after correction) sometimes as large as 15 per cent was observed when the boundary approached the bottom of the cell. This abnormal decrease is tentatively explained on the assumption that the cell might not have been in perfect alignment and that some material escaped from the solution by sliding down along the wall to the bottom.

The ratio of the area under the apoferritin boundary to the total area is equal to the percentage of free apoferritin provided two corrections are made; first, the areas are to be multiplied by the factor r_b^2/r_m^2 (r_b and r_m are the distances from the center of rotation to the boundary and the meniscus respectively), and second, they should be brought to a common refractive index increment. Refractive index increments were therefore measured with a Pulfrich refractometer in a constant temperature room at 25°, on dialyzed solutions of apoferritin, ferritin, and iron hydroxide. The results have been summarized in Table I. The precision of the values of $\Delta n/c$ is

TABLE I
Refractive Index Increments of Apoferritin and Iron Hydroxide

Substance	Concentration	Δn	$\frac{\Delta n}{c}$
	<i>per cent</i>		
Apoferritin.....	1.11	0.00192	0.00173
Ferritin (17.7% iron).....	1.49	0.00273	0.00183
Iron hydroxide.....	0.281	0.00061	0.00218

about 1 or 2 per cent. Assuming the increment of ferritin to be an additive function, one can calculate its increment from those of apoferritin and iron hydroxide, since the concentration in iron is known. The calculated value 0.00185 is in good agreement with the experimental one 0.00183.

The results of two representative experiments to determine the amount of free apoferritin from area relationship have been summarized in Table II.

The figures in Column 2 indicate the time at which the exposures were made after the centrifuge had reached maximum speed. The figures in Columns 5 and 6 have been obtained by multiplying the values in Columns 3 and 4 by the factors r_b^2/r_m^2 . The total areas in Column 7 have been obtained by multiplying the values in Column 5 by the refractive index ratio 173/183 from the data in Table I.

The second method used to determine the percentage of free apoferritin in ferritin was as follows: A dialyzed solution of ferritin of known concentration was centrifuged in a separation cell. After it was visually observed that all of the iron complex had sedimented below the partition, the centrifuge was stopped and an exposure was made at low speed just before the rotor came to rest. From the position of the boundary of free apoferritin

and the dimensions of the cell (1.2 cm. cell with an aperture of 4°) the amount of free apoferritin left in the upper chamber after centrifugation was given by the following equation.

$$\text{Gm. apoferritin} = \frac{4}{380} 1.2\pi(r_p^2 - r_b^2) \frac{r_m^2}{r_b^2} C$$

where r_p , r_b , and r_m are the distances from the center of rotation to the partition, boundary, and meniscus respectively, and C is the unknown concentration (gm. per cent) of free apoferritin in the original solution.

TABLE II
Determination of Free Apoferritin from Area Relationship

Experimental conditions (1)	Time (2)	Area		Area corrected for decrease in concentration		Area corrected for refractive index		Relative amount of apoferritin (9)	Absolute amount of apoferritin (10)
		Total (3)	Apo- ferritin (4)	Total (5)	Apo- ferritin (6)	Total (7)	Apo- ferritin (8)		
	min.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	per cent	gm. per cent
Iron content 17.7%, concentra- tion of solution 1.5%, cell 12 mm. thick, 43,200 R.P.M., 25.5° , 35° slit angle	2	0.70		0.79		0.74 _s			
	4	0.67		0.78 _s		0.74			
	6	0.64		0.78		0.74			
	13		0.17		0.19		0.19	26	0.45
	18		0.16		0.175		0.175	24	0.42
	34		0.15		0.175		0.175	24	0.42
	65		0.14		0.175		0.175	24	0.42
Iron content 22.7%, concentra- tion of solution 0.46%, cell 12 mm. thick, 32,400 R.P.M., 20.5° , 45° slit angle	20	0.30	0.063	0.36	0.067	0.34	0.067	20	0.11

The fluid of the upper chamber was removed with a hypodermic needle, the chamber rinsed several times to remove all the material, and the dry weight of total fluid of the upper chamber was determined. C was calculated by $C = 100W/V$, where W is the total dry weight and V the factor

$$\frac{4}{380} 1.2\pi(r_p^2 - r_b^2) \frac{r_m^2}{r_b^2}$$

Since the capacity of the separation cell was small (≈ 0.75 cc.), two cells were used at the same time. The second cell was placed in the hole normally occupied by the dummy cell. As a rule, two cells could not be filled exactly to the same height and a double pattern was thus observed, but no

difficulty was encountered in locating the position of each boundary. An analysis was made with the solution used in the first experiment summarized in Table II. Two sedimentation runs were made, each one with two cells, and the contents of the upper chambers pooled together. The total volume of solution was 2.94 cc. and the values of V and W were 0.43 cc. and 0.00152 gm. respectively.

Since the ferritin concentration of the original solution was 1.5 per cent, the percentage of free apoferritin in ferritin was then

$$\frac{10 \cdot W}{1.5V} = \frac{15.2}{1.5 \times 0.43} \approx 24\%$$

As mentioned earlier in the paper, the agreement between the two methods is as good as could be expected.

Precision Measurements of Rates of Sedimentation As Illustrated by Studies on Apoferritin

A few years ago, Lauffer and Stanley (9), in collaboration with three other laboratories equipped with ultracentrifuges, determined the probable error in the value of a constant of sedimentation, using the fast sedimenting molecule of bushy stunt virus as material. Even in a given laboratory, variations in the constant s from run to run were much larger than expected, discrepancies as large as 10 per cent being observed with the same material. No conclusion was drawn as to whether there was some unknown cause of error or whether changes had occurred in the material investigated. The question of the accuracy of the ultracentrifuge, as at present used, thus remained open. Taking advantage of the great stability of apoferritin in solution, the writer decided to make a study of the rate of sedimentation of this protein, with the maximum precision available, as a partial answer to this question.

The ultracentrifuge used was that described in earlier work (10, 11). The mean effective radius of the rotor, *i.e.* the distance between the center of rotation and the middle of the cell, is 6.5 cm. All experiments were carried out under a vacuum of 10^{-3} mm. of Hg in the chamber containing the rotor.

The accuracy of the determination of a rate of sedimentation is limited by the sensitivity of the optical arrangement used, by the controls attained in the speed of rotation, and the temperature. In the optical measurements the Svensson-Philpot schlieren method was used. A few determinations were also made with the scale method of Lamm. When the first method was used, eight to ten successive exposures were taken on the same plate, as this procedure greatly facilitates accurate measurements of the successive positions of the boundary. The results of a representative experiment can

be seen in Fig. 2, *c*. The schlieren and scale methods gave identical results within the limit of error which was estimated to be ± 0.3 per cent.

The speed of rotation was measured with the aid of a Strobotac. To increase the precision, the Strobotac was used on the "line" connection and the frequency of the periodic flashes was thus equal to the "line" frequency which is maintained constant to high accuracy. All experiments were made at speeds which were multiples of 60 revolutions per second, the rotating unit appearing motionless when its speed of rotation is an exact multiple of the frequency of the flashes. The value of the multiple of the 60 cycle frequency corresponding to the speed of rotation was determined with the Strobotac on the regular scale. Two or more successive sub-multiples of the speed of rotation were measured. With these data the value of the multiple of the 60 cycle frequency corresponding to the speed of rotation was found. It is estimated that the speed was maintained constant within less than 0.1 per cent.

The temperature of the rotor was measured with an accuracy of $\pm 0.05^\circ$ by an iron constantan thermocouple mounted on a rod which could move vertically through an air-tight bearing; this arrangement permitted temperature measurements without releasing the vacuum. The temperature of the rotor was taken before and after an experiment by bringing the couple in contact with the rotor. The mean temperature is the one reported in Table III. Since all experiments were made at speeds between 43,200 and 54,000 R.P.M., there was an increase in temperature of the rotor from 0.1 – 1° during a run, in spite of the fact that the temperature 2 cm. above the rotor was kept 5 – 10° lower than that of the rotor itself. The temperature of the solution was assumed equal to that of the rotor but it may possibly have differed from it by a few tenths of a degree. This would explain the slight variations in the value of s_{20}^0 which were, however, never larger than 1.5 per cent from run to run.

The temperature control seems to be at present the limiting factor in the accuracy of a rate of sedimentation, on account of the large temperature coefficient of the viscosity of aqueous solutions, which is about 2.5 per cent per degree.

This study had also the special object of testing the formula for the variation of the constant of sedimentation with the temperature. This test appeared particularly desirable in view of the fact that it has recently been proposed by MacInnes (6) to adopt 0° as the standard temperature for the measurement of physical properties of proteins, especially as electrophoresis experiments must be made at or near that temperature. The ultracentrifuge used in this investigation seemed particularly suitable for such tests, since it is equipped, as previously described (11), with a cooling system permitting measurements of sedimentation rates at constant tem-

TABLE III

Rate of Sedimentation of Apoferritin

The abbreviations in Column 4 stand for the following: 0.1 Ac = 0.1 M acetate buffer + 1 per cent NaCl, pH 4.6; $\delta_{20} = 1.0134$; $\delta_0 = 1.0150$; $\eta_{20} = 0.01079$; $\eta_1 = 0.01846$. 0.05 Ac = 0.05 M acetate buffer + 1 per cent NaCl, pH 4.6; $\delta_{20} = 1.0080$; $\delta_0 = 1.0106$; $\eta_{20} = 0.01041$; $\eta_1 = 0.01782$. Ver. = 0.025 M veronal buffer + 1 per cent NaCl, pH 7.6; $\delta_{20} = 1.0073$; $\eta_{20} = 0.01031$. All densities are based on weights *in vacuo*.

Experiment No.	Velocity	Concentration	Medium	Temperature	s_1	s_0°
(1)	(2)	(3)	(4)	(5)	(6)	(7)

Horse apoferritin						
	R.P.M.	per cent	per cent	°C.	Svedberg units	Svedberg units
1	43,200	0.8	1.0 NaCl	22.8	17.5	16.9
2	43,200	0.28	0.1 Ac	21.5	15.8 _s	17.1
3	43,200	0.28	0.1 "	20.7	15.6	17.1 _s
4	43,200	0.28	0.1 "	20.4 _s	15.7 _s	17.4
5	43,200	0.28	0.1 "	18.5	14.9	17.1 _s
6	43,200	0.28	0.1 "	20.9	15.9	17.3
7	43,200	0.28	0.1 "	20.5	15.4 ₇	17.0 _s
8	43,200	0.14	0.1 "	20.1	15.6	17.3 _s
9	43,200	0.14	0.1 "	21.2	{ 15.8 _s 15.8 _s *	{ 17.2 _s 17.2 _s *
10	43,200	0.14	0.1 "	19.5	15.1	17.1
11	46,800	0.07	0.1 "	19.5	15.3 ₇	17.4
12	43,200	0.07	0.1 "	20.2 _s	15.9 _s	17.7
13	43,200	0.28	0.1 "	2.4	9.4 ₇	16.8
14	43,200	0.28	0.1 "	3.8	9.9 _s	16.8
15	50,400	0.28	0.1 "	1.2 _s	9.1 _s	16.9
16	50,400	0.14	0.1 "	2.3	9.7 ₁	17.3
17	50,400	0.14	0.1 "	3.1 _s	9.8 _s	17.1 _s
18	50,400	0.14	0.1 "	1.4	9.2	16.9
19	50,400	0.14	0.1 "	1.1 _s	9.0 _s	16.8
20	54,000	0.07	0.1 "	1.3	9.3 _s	17.1 _s
21	54,000	0.07	0.1 "	1.0	9.2 ₁	17.1
22	50,400	0.07	0.1 "	1.8	9.7 _s	17.5 _s
23	43,200	0.54	0.05 "	20.2	16.1 _s	16.9 _s
24	43,200	0.27	0.05 "	20.1 _s	16.2 _s	17.1 _s
25	43,200	1.09	Ver.	20.5	15.7 _s	16.3
26	43,200	0.27	"	20.0	17.1	17.9 _s
27	43,200	0.27	"	20.0	17.1 _s	18.0
28	43,200	0.11	"	21.5 _s	17.6 _s	17.9
29	54,000	0.11	"	2.2	10.7	17.9
30	54,000	0.11	"	2.3	10.6	17.7

Human apoferritin						
31	46,800	0.5	0.05 Ac	19.4	15.8	17.0

TABLE III—*Concluded*

Experiment No.	Velocity	Concentration	Medium	Temperature	s_t	s_{20}^0
(1)	(2)	(3)	(4)	(5)	(6)	(7)
Dog apoferritin						
	R.P.M.	per cent	per cent	°C.	Svedberg units	Svedberg units
32	46,800	0.11	0.05 Ac	20.4	16.8 _s	17.7
33	43,200	0.11	0.05 "	20.3 _s	17.1 _s	18.0 _s
34	43,200	0.11	0.05 "	21.2 _s	17.1 _s	17.8
35	54,000	0.11	0.05 "	3.0 _s	10.8 _s	17.8 _s
36	54,000	0.11	0.05 "	4.9	11.3 _s	17.8 _s
Horse heavy globulin						
37	43,200	0.19	0.1 Ac	20.2	17.7	19.7
38	43,200	0.19	0.1 "	20.2	17.6	19.5 _s
39	54,000	0.19	0.1 "	2.1	10.7	19.1
40	50,400	0.19	0.1 "	2.7	10.8	19.0

* Scale method.

peratures around 0°. The data obtained on apoferritin are outlined in Table III. It will be noticed in Column 5 of Table III that determinations were made near 20° and 0°. If the molecules are not so small as to require the maximum possible velocity when the experiment is carried out at room temperature, the determination of a rate of sedimentation at a lower temperature is more accurate. The time of the experiment can be made the same by increasing the speed and the effect of diffusion is smaller at the lower temperature. The advantage of low temperature is especially marked for very dilute solutions. The increase of the sharpness of the peaks at 0° over those obtained at 20° is well illustrated in Fig. 2, *d* and *e*. The time in both of these experiments was the same. The formula used for reducing the sedimentation constants, s_t , to a standard basis, s_{20}^0 (water at 20° being the usual reference solvent), is as follows:

$$s_t = s_{20}^0 \frac{\eta_{20}^0 (1 - V_t \rho_t)}{\eta_t (1 - V_{20} \rho_{20}^0)}$$

Values of η_t and ρ_t were determined with an Ostwald viscosimeter and a quartz pycnometer, respectively. The variations of s_t between 1° and 20° are large, about 60 per cent. However, it can be seen from Column 6 of Table III that the reduction formula is entirely adequate in the case of dog apoferritin. The same value of s_{20}^0 is obtained whether sedimentation was carried out at 3° or 20°.

For horse apoferritin there is a small but consistent difference; the values

s_{20}^0 calculated from experiments at low temperatures are smaller by about 1 per cent than the values obtained at 20°.

On account of the low value found for the asymmetry factor $f/f_0 = 1.14$, the sedimentation constant is rather insensitive to change in concentration, as can be seen from Columns 3 and 7 of Table III. However, the average value found for the constant s_{20}^0 with 0.25 per cent solutions is on the average smaller by 2.3 per cent than that determined with 0.07 per cent solutions. The following values extrapolated for zero concentration were obtained from experiments made with horse apoferritin at about 20° and at pH 4.6: $s_{20}^0 = 17.6$ Svedberg units, $s_0^0 = 10.2$ Svedberg units. The corresponding values obtained from measurements at about 1° were $s_{20}^0 = 17.4$ Svedberg units, $s_0^0 = 10.1$ Svedberg units. Columns 4 and 7 of Table III indicate that the pH of the solution has only a small effect on the rate of sedimentation. In veronal buffer at pH 7.6 the constant $s_{20}^0 = 18.0$ Svedberg units is slightly greater than at pH 4.6.

Molecular Weight of Apoferritin—For the determination of the molecular weight of a substance its diffusion constant and specific volume are needed as well as the sedimentation constant.

Diffusion measurements were carried out at 1° in the Tiselius apparatus, as already described (10, 11). Measurements were made with a 0.565 per cent solution in 0.05 M sodium acetate buffer and 1 per cent sodium chloride at pH 4.6. The diffusion constant was calculated from the relation $D = s^2/4\pi t H_{\max}^2$, where s is the diffusion area in sq. cm., t the time in seconds, and $H_{\max}$ the maximum height of the curve in cm. The patterns of both arms of the cell were studied for the computation. When values of $1/H^2$ were plotted against time, good straight lines were obtained. The value found for the tangent ($1/H^2 \times 1/t$) was $1.29_8 \times 10^{-6}$ for the boundary of one arm and $1.30_4 \times 10^{-6}$ for the other. The areas under the curves were found constant, the average value of eleven patterns being 1.38 sq. cm. for one boundary and 1.34 sq. cm. for the other. The average value of 1.36 sq. cm. was chosen for computation. Thus the diffusion coefficient D at 1° equals 1.91×10^{-7} . The value of the diffusion constant D_{20}^0 (in water at 20°) is found from the relation

$$D_{20}^0 = D_1 \frac{\eta_1 293}{\eta_{20}^0 273} = 3.61 \times 10^{-7}$$

in which η is the viscosity. Another diffusion experiment on a different sample of apoferritin made at 1° in a 0.1 M sodium acetate buffer and 1 per cent sodium chloride at pH 4.6 gave $D_{20}^0 = 3.5 \times 10^{-7}$. This value is, however, considered to be less accurate, since the experimental controls were not as complete.

The specific volume of apoferritin was calculated from accurate deter-

mination of the density of solutions of known concentration. To insure adequate precision, measurements were made in a quartz pycnometer of about 30 cc. capacity. The following values of the density, δ , were found for a dialyzed solution of apoferritin containing 0.0113 gm. of protein per cc.

$\delta_{20}^{\circ} = 1.00109_6$, 1.00112_2 , and 1.00110_1 ; $\delta_1^{\circ} = 1.00293$ (based on weights *in vacuo* and δ_{20}° water = 0.99823).

The calculated average values $V_{20}^{\circ} = 0.747$ and $V_1^{\circ} = 0.738$ were chosen for computation. The value 0.747 is in close agreement with the value 0.749 given for proteins by Svedberg. (Svedberg's measurements were not extended below 16° but by applying his temperature coefficient an extrapolated value of $V_1^{\circ} = 0.739$ is obtained.) The molecular weight was calculated from the usual formula

$$M = \frac{RTs}{D(1 - V\rho)}$$

Two sets of values may be chosen for s , D , V , and ρ , either s_{20}° , D_{20}° , V_{20}° , and ρ_{20}° or s_1 , D_1 , V_1° , and ρ_1 .

The following values were calculated,

$$M = \frac{8.31 \times 293 \times 17.6 \times 10}{3.61(1 - 0.747 \times 0.9982)} = 467,000$$

and

$$M = \frac{8.31 \times 274 \times 9.85 \times 10}{1.91(1 - 0.738 \times 1.0106)} = 462,000$$

from data obtained at 20° and 1° , respectively. It is gratifying to find that the value found for the molecular weight is the same whether calculated from measurements at room temperature or near 0° , especially if it is considered that constants of sedimentation are about 60 per cent larger at room temperature than at 0° . The asymmetry factor calculated from s_{20}° and D_{20}° was found to be $f/f_0 = 1.14$, thus showing that the molecule is not very asymmetric. On the basis of Perrin's formula the axis ratio is about 3.

I wish to thank Dr. D. A. MacInnes for helpful suggestions in the preparation of this article.

SUMMARY

Ferritin, a crystallized protein with 20 per cent iron, and apoferritin, a crystallized iron-free protein obtained from ferritin, were investigated in the ultracentrifuge. It was found that ferritin is not a definite molecular species but consists of a mixture of a complex of apoferritin-iron hydroxide

and about 25 per cent free apoferritin. The mass of these complex particles is not uniform. However, apoferritin is a very homogeneous protein. A study of the sedimentation rate of apoferritin obtained with all the precision now available showed that the sedimentation constant of apoferritin is $s_{20}^0 = 17.6$ Svedberg units. This value is significantly lower than that found for the normal heavy globulin of horse serum which has a sedimentation constant $s_{20}^0 = 19.5$ Svedberg units. The molecular weight of apoferritin, whether obtained from measurements at 1° or at room temperature, was found equal to 465,000. The asymmetry factor is $f/f_0 = 1.14$, showing that the molecule has an asymmetry of the same order as egg albumin. The sedimentation constants of horse and human apoferritin are identical; that of dog apoferritin is 3 per cent greater.

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LETTERS TO THE EDITORS

SEPARATION OF CYTOCHROME OXIDASE INTO TWO COMPONENTS

Sirs:

An ultracentrifugal study of cytochrome oxidase prepared according to the new procedure¹ seemed to indicate the participation of two components in the enzymatic oxidation of cytochrome *c* by molecular oxygen. The two components differ markedly in their heat stability. Component I loses 80 per cent of its activity in 15 minutes at 50°, while Component II may be kept for the same length of time in boiling water without loss of activity. Component I is prepared by centrifugation of the diluted oxidase¹ for 2 hours at 10,000 R.P.M. The sediment contains an excess of Component I together with a small quantity of Component II. No further purification of Component I has as yet been attempted. Since the component is insoluble, it may be anticipated that ultrasonic disintegration will be the first step required. Component II is obtained by incubating undiluted oxidase in boiling water for about 3 minutes. The great bulk of heat-denaturated protein is separated by centrifugation, washed with ammonium buffer of pH 9, and discarded. The supernatant solution combined with the wash liquid contains Component II. After centrifugation for 2 hours at 10,000 *g* a water-clear, slightly yellow solution is obtained, which contains 72 per cent of the original activity. Despite its unusual heat stability Component II also seems to be a protein. It is precipitated by ammonium sulfate, is not dialyzable, and no loss of activity was caused by dialysis for 15 hours at 0° against 0.004 *M* pyrophosphate of pH 7.9. Separation of the two components affects their stability to a great extent. Component II in ammonium buffer of pH 9 loses 40 per cent of its activity upon standing overnight at 0°, while the unsplit oxidase under similar conditions remains unchanged for several weeks. The two components herein described are required for the oxidation of cytochrome *c* by molecular oxygen. They are not identical with the activating principle discovered by Stern and Melnick,² which seems to play a rôle in the reduction of cytochrome *c* by succinate.³ The enzymatic activity of the unsplit oxidase and of the separated and of the recombined components in transferring molecular oxygen to cytochrome *c* is demonstrated in the table.

¹ Haas, E., *J. Biol. Chem.*, **143**, 481 (1943).

² Stern, K. G., and Melnick, J. L., *Nature*, **144**, 330 (1939).

³ Straub, F. B., *Z. physiol. Chem.*, **272**, 219 (1942).

Temperature 25°; gas phase, air. 2.5 ml. of 0.05 M phosphate buffer of pH 7.1 + 1.0 mg. of cytochrome *c* + 3.0 mg. of hydroquinone.

	Experiment 1 0.10 ml. unsplit oxidase	Experiment 2 0.10 ml. Component I	Experiment 3 0.20 ml. Component II	Experiment 4 0.10 ml. Component I 0.20 " " Component II
	Oxygen uptake			
min.	c.mm.	c.mm.	c.mm.	c.mm.
15	68	15	0	56

The small oxygen uptake in Experiment 2 is probably due to the contamination of Component I with a small amount of Component II. A comparison of Experiments 1 and 4 indicates that the recombined components represent a considerable fraction of the original activity. The fact that two components are required for the reaction between molecular oxygen and cytochrome *c* suggests that Warburg's oxygen-transferring enzyme and cytochrome oxidase are not identical but represent two individual enzymes.

*Department of Chemistry
University of Chicago
Chicago*

ERWIN HAAS

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